

**DEVELOPMENT OF PHOTSENSITIVE LIPOSOMES FOR THE CONTROLLED
RELEASE OF DRUGS**

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Doctor of Philosophy

ASTON UNIVERSITY

April 2015

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Thesis Summary

The facility to controlled triggered release from a “cage” system remains a key requirement for novel drug delivery. Earlier studies have shown that Bis-Azo PC based photosensitive liposomes are beneficial for drug delivery. Thus, the aim of this project was to develop photosensitive liposomes that can be used for the controlled release of drugs through UV irradiation, particularly therapeutic agents for the treatment of psoriasis.

Bis-Azo PC was successfully synthesized and incorporated into a range of liposomal formulations, and these liposomes were applied for the controlled release of BSA-FITC. Bis-Azo PC sensitized liposomes were prepared via interdigitation fusion method. IFV containing optimum cholesterol amount in terms of protein loading, stability and photo-trigger release of protein was investigated. Further studies investigated the stability and triggered release of the HMT from IFV. Finally, permeation behavior of HMT and HMT-entrapped IFV through rat skin was examined using Franz cell.

Results from protein study indicated that the stable entrapment of the model protein was feasible as shown through fluorescence spectroscopy and maximum of 84% protein release from IFV after 12 min of UV irradiation. Moreover, stability studies indicated that IFV were more stable at 4 °C as compared to 25 °C. Hence, DPPC:Chol:Bis-Azo PC (16:2:1) based IFV was chosen for the controlled release of HMT and these studies exhibited that photo-trigger release and stability data of HMT-entrapped IFV are in line with the protein results. Franz cell work inferred that HMT-entrapped IFV attributed to slower skin permeation as compared to HMT. CLSM also demonstrated that HMT can be used as a fluorescent label for the *in vitro* skin study. Overall, the work highlighted in this thesis has given useful insight into the potentials of Bis-Azo PC based IFV as a promising carrier for the treatment of psoriasis.

Key Words: Bis-Azo PC, IFV

Dedication

To all family members, especially my father ‘Umaji Kisan Bhujbal’ , my sweet mother ‘Surekha Umaji Bhujbal’ and Tejas Umaji Bhujbal whom without their support, I would not have been able to finish this hard work. My dear parents, without your motivation and moral support, I couldn’t complete this journey. You boost my confidence, prayed for me and backed me up with all resources. I simply don’t have words to express my love and gratitude to you.

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Abbreviations

ANOVA	Analysis of variance
Bis-Azo PC	1, 2-bis (4-(n-butyl) phenylazo-4'-phenylbutyroyl) phosphatidylcholine
BSA	Bovine serum albumin
BSA-FITC	Bovine serum albumin-Fluorescein Isothiocyanate
Chol	Cholesterol
cm	Centimetre
¹³ C NMR	Carbon 13 nuclear magnetic resonance spectroscopy
CLSM	Confocal laser scanning microscopy
°C	Degree Celsius
D ₂ O	Deuterium oxide
DMAP	4-(N, N-dimethylamino) pyridine
DPPC	2-dipalmitoyl-sn-glycero-3-phosphocholine
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
EE	Entrapment efficiency
FDC	Franz diffusion cell
FCC	Flash column chromatography
FTIR	Fourier-transform infrared spectroscopy
hν	High voltage
h	Hour
HMT	4'-(6-Hydroxyhexyloxy) methyl-4, 5', 8-trimethylpsoralen
¹ H NMR	Hydrogen nuclear magnetic resonance spectroscopy
IR	Infra-red spectroscopy
IFV	Interdigitation fusion vesicles

LUV	Large unilamellar vesicles
LUVETs	Large unilamellar vesicles by extrusion method
MS	Mass spectrometry
MLV	Multilamellar vesicles
mg	Miligram
mL	Mililitre
mV	Milivolt
Min	Minutes
µg	Microgram
µL	Micro litre
µm	Micro metre
µmoles	Micro moles
NIR	Near-infrared
nm	Nanometer
ppm	Parts per million
PBS	Phosphate buffered saline
Glycero-PC	Glycerophosphatidylcholine
PC	Phosphatidylcholine
PDI	Polydispersity Index
PUVA	Psoralen along with Ultra-Violet A
RBF	Round bottom flask
Rf	Retention factor
TLC	Thin layer chromatography
Sec	Seconds
SD	Standard deviation

SUV	Small unilamellar vesicles
SC	Stratum corneum
T _m	Transition temperature
UV	Ultraviolet
UVA	Ultraviolet A light
UVB	Ultraviolet B light

CHAPTER 1

INTRODUCTION

Chapter 1-Introduction

1.1. Introduction

Light is one of the best known innocuous "materials" available to man due to its wavelength and intensity. Light intensity and wavelength can be regulated by the use of coloured filters, and fabricated photo masks or lasers which not only allocate for fabrication of complex features but also cover areas with resolution as small as approximately 1 μm (Katz and Burdick, 2010). A recent advance in confocal microscopy has empowered a remarkable increase in resolution with three dimension control (Helmchen and Denk, 2005; Zipfel et al., 2003). Technology has allowed light to be employed in non-invasive deep tissue imaging modalities like diffuse optical tomography and photoacoustic imaging (Charrois and Allen, 2003; Ishida et al., 1999). However, employing the same strategy towards drug delivery applications has not reached the same level. Light could be employed to accomplish drug delivery either by exploiting photophysical or photochemical principles. Photochemical means of drug delivery works as a result of photochemical reactions that take place in the drug delivery vehicle. The photochemical transformation can be planned in such a way that the stability of delivery vehicle is effectively destroyed, imposing molecular reorganization in the drug delivery vehicle. This reorganization method can be potentially consumed to let the leakage of drug presented in the interior of the drug carrier. The advantage of a photochemical transformation is that it can be designed to occur by a two-photon excitation process that offers better spatiotemporal control. Yet another potential advantage of the photochemical process of drug delivery is that the delivery vehicles are usually composed of only organic molecules that can be specifically metabolized by various enzymes in the body for elimination once the supramolecular assembly is destroyed after drug delivery (Karanth and Murthy, 2007; Torchilin, 2005).

1.1.1. Light based therapies

The success of light based therapies is completely reliant on the selection of ample light sources that can deeply penetrate the tissues for drug delivery and therapeutic applications. The bulk of light- responsive biomaterials are responsive in the UV spectral range which is not limiting step in an *in vitro* surroundings, and may be useful for different applications. But, these types of materials are not useful for *in vivo* surroundings due to the poor penetration of UV light deeply into tissues. The reason behind poor penetration is the scattering and presence of endogenous light absorbers, such as oxy- and deoxy-hemoglobin, lipids and water (Klohs et al., 2008). In order to hinder tissue-penetration concerns, light based therapies have been more widely used for superficial areas such as skin and oral treatments (Donnelly et al., 2008; McCoy et al., 2007) than deeper skin furrows. However, the preferred choice for *in vivo* surrounding therapeutics is near-infrared (NIR)

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responsive biomaterials. NIR light ranges from 700 nm to 2500 nm and penetrates deeply into the human skin and blood up to the 1 cm (Weissleder and Ntziachristos, 2003). Nowadays advances in two-photon microscopy and lasers as well as the development of NIR active molecules have played important roles in the treatment of cancer and bladder diseases (Gurfinkel et al., 2004).

Several nanocarriers are used in light based therapy, particularly, lipid based nanocarriers have become popular tool due to several reasons (Demirbag and Kucukturhan, 2011). Firstly, they protect the drug from lymphatic system. Secondly, they protect the non-target tissues from toxic effects of the bioactive agent. Thirdly, lipid based nanocarriers can be easily metabolized and excreted from the body without leaving any by-products behind. Lipid based nanocarriers are prepared by simple methods and exist in different forms and ranges of size. Moreover, they have the ability to encapsulate both water soluble and lipid soluble materials (Arias et al., 2011). Fig 1.1 illustrates the different forms of lipid based nanocarriers.



Fig 1.1: Different forms of lipid based nanocarriers [taken from (Dijkhuizen, 2011)].

Based on the preparation method, lipid based nanocarriers may range from 10 to 1000 nm in size. Drug loading of lipid based nanocarriers can be performed by several techniques. In order to avoid rapid clearance from the circulation via cells of the reticulo-endothelial system (RES), lipid based nanocarriers can be modified into hydrophilic shell, i.e. Polyethylene glycol (PEG)-lipid based nanocarriers (Allen and Chonn, 1987; Lasic et al., 1992). The size of tissue targeting of lipid based nanocarriers can be increased through introduction of ionisable groups which have a specific affinity towards specific tissue due to charge or size (Tam et al., 2013). Lipid based nanocarriers can exist in different forms which are presented in Fig 1.1. Among all these nanocarriers, liposomes are the oldest and the most common type used for biomedical applications (Liu et al., 2005a; Torchilin, 2003).

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1.2. Liposomes

Liposomes, also known as fatty acid vesicles (Walde, 2006), are artificial spherulites that emerge highly ordered phospholipids when hydrated in aqueous media. Liposomes originated in the work of Alec D Bangham and co-workers (Bangham and Horne, 1964) at the Babraham Institute in Cambridge. While studying phospholipids with the help of electron microscope, they discovered bilayer lipid structure by adding negative stain to dry phospholipid. Initially they termed these materials as ‘smecticmesophase system’. Later on Gerald Weissmann renamed them as liposomes and published a paper on diffusion of univalent ions across the lamellae of swollen phospholipids (Bangham et al., 1965). However in the early 1970s, for the first time, liposomes were considered as one of the safest carrier systems for drugs, antigens and enzymes (Gregoria.G and Ryman, 1971; Gregoriadis, 1993). Other early pioneers (Alahari et al., 1998) proved that liposomes could change the *in vivo* distribution of the entrapped drugs. From then on, liposomes have been used to deliver drugs for cancer and other diseases (Gregoriadis and Florence, 1993). Although it has been generally accepted that the use of liposomes with proper composition should result in increased drug transport *in vitro* and *in vivo*, many questions arise about the mechanism of action of these vesicular formulations

1.2.1. Mechanism of action

The term liposome originated from the Greek words: “lipo” which means fat and “soma” which means body. Liposomes are composed of a hydrophobic bilayer membrane which entraps an aqueous phase inside (Gregoriadis et al., 2002). As a result, during liposomal formulation, hydrophilic substances or drugs dissolved into this aqueous phase, and due to lipid bilayer coating these hydrophilic materials cannot bypass the membrane and remain inside the lipid bilayer. Hydrophobic materials or drugs can also be dissolved into the bilayer membrane. In this manner liposomes are able to carry both hydrophilic and lipophilic materials (Chrai, 2002). In order to deliver these types of materials to a target site, the lipid bilayer of liposomes should be able to readily fuse with the lipid bilayer of cell membrane. So by direct cell fusion, liposomes can deliver their contents (Kamps and Scherphof, 2003). Apart from cell fusion, liposomes also deliver their contents by diffusion mechanism. For instance, in case of pH sensitive liposomes, inner and outer pH values play important role and charged drugs can easily come out from the liposome membrane by pH-dependent diffusion mechanism (Ishida et al., 2006). In some cases, liposomes are used to target the endocytosis process by macrophagocytes. In that macrophages engulf liposomes with a particular size and this process releases the liposomes drug contents (Scherphof and Kamps, 2001; Scherphof and Kamps, 1998). Moreover, liposomes directly discharge their contents through

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adsorption mechanism (Kamps and Scherphof, 2003), and also deliver DNA material into the host cell membrane by transformation or transfection (Daemen, 1998). Fig 1.2 represents the possible ways by which liposomes can release their contents.



Fig 1.2: Possible ways by which liposomes can release their contents [taken from (Kamps and Scherphof, 2003)].

1.2.2. Morphological aspects of liposomes

Liposomes are termed as an ideal system for the study of the properties of contemporary biomembranes due to their close resemblance to biological membranes and, in studies examining the appearance, performance and the origin of cellular life (Monnard and Deamer, 2001; Mozafari et al., 2004; Pozzi et al., 1996). Just like biomembrane, liposomes are made up of phospholipids. These phospholipids contain phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserines (PS) (Chapman, 2006). Phospholipids are amphiphiles which possess a hydrophilic head group and a hydrophobic tail group (Fig 1.3). The hydrophobic tail group is

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composed of two fatty acid chains (10-30 carbon length) and the hydrophilic head group usually consists of negative charged phosphate group along with polar groups. As being amphiphiles in nature, structures like liposomes form instantly (Fig 1.3) when placed in water, the hydrophilic head groups are easily attracted to water and assemble to form a surface facing the water whereas the hydrophobic tail groups are repulsed by water, and rearrange to form a surface away from the water (Frézard, 1999). The instant formation of liposomes not only forms the closed lipid bilayers within a lamellar arrangement (where hydrophobic tail group are sandwiched between the hydrophilic head group of phospholipids) but also passively entraps the aqueous media as phospholipids are placed in water. Because of this, the solutes such as drugs, enzymes, proteins, antibiotics and nucleic acids presented in polar solvent can easily be entrapped within these liposomes. As a result the entrapped materials are easily protected from surroundings and delivered to the appropriate site in order to give therapeutic effect (Gregoriadis, 1976). In short, liposome can encapsulate and efficiently deliver both hydrophilic and lipophilic substances (Akbarieh et al., 1992; Fielding, 1991) and may be used as a non-toxic vehicle for insoluble drugs (Lidgate et al., 1988).

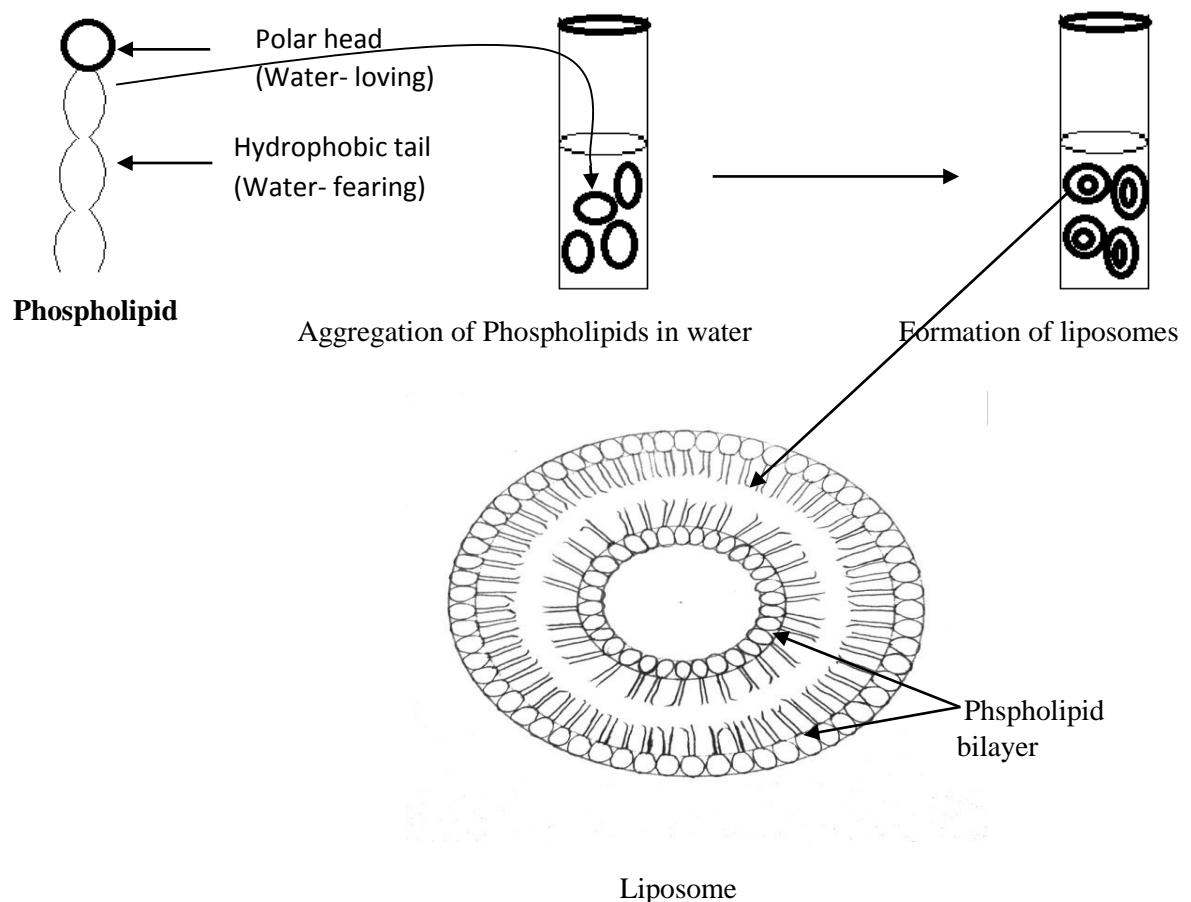


Fig 1.3: Schematic representation of vesicle formation from phospholipid.

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Liposomes are categorized by their number of lamellae, lipid content and particle size distribution. All these factors dictate their stability and interaction capabilities.

Based on the structure, they are classified into multilamellar vesicles (MLV), small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), oligolamellar vesicles (OLV) and multivesicular vesicles (MMV) (O'Doherty, 2004) (Fig 1.4). Both small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) have one lipid bilayer. Therefore they are termed as unilamellar vesicles (ULV) (Perezsoler, 1989). Due to one lipid bilayer ULV enclose large central aqueous media. These characteristics are ideally suited for the encapsulation of water soluble agents (Harrington et al., 2002).

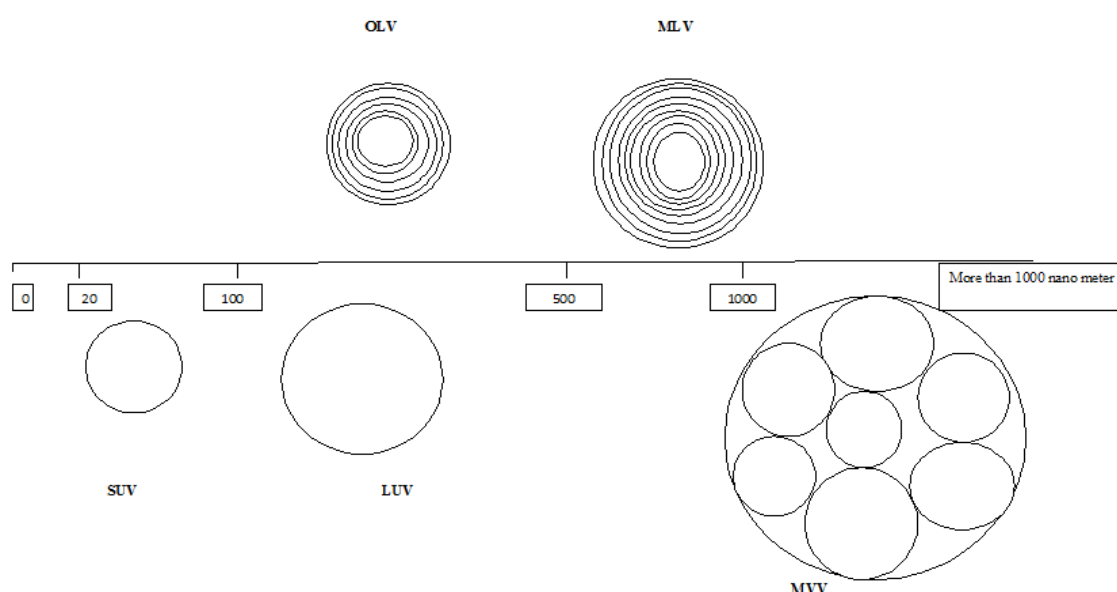


Fig 1.4: Schematic representation of types of liposomes based on their size [adapted from (Liposome Quotes, (2013)).

a) Multilamellar vesicles

MLV have diameters above 500 nm and, normally consist of five to twenty lipid bilayers. Because of bigger size and concentric phospholipid bilayer membranes, MLV internally acquire small aqueous compartment and higher lipid/aqueous distribution ratio (O'Doherty, 2004). Therefore they are good for encapsulation of lipid soluble agent i.e. hydrophobic drugs (Harrington et al., 2002). These liposomes are very useful in the case of cancer therapy as most of the cytotoxic drugs are hydrophobic (Leylandjones, 1993). MLV are easily cleared by Reticuloendothelial System (RES) and useful for targeting cells of RES (Sharma and Sharma, 1997). They are very stable compared to SUV. During formation step, MLV reorganize themselves in random order which is why the size of MLV cannot be controlled (Gregoriadis et al., 2002).

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b) Small unilamellar vesicles

SUV are vesicles that have size between 20 to 100 nm and considered as the smallest vesicles compared to all other vesicles. They have a single bilayer structure enclosing small aqueous core. SUV capture limited amount of hydrophilic macromolecules due to higher curvature and lower lipid/aqueous distribution ratio (Liu, 2000). However expert in Jena University (Decker et al., 2013) used SUV for the delivery of lipophilic drugs. SUV are not easily cleared by RES and have long half-life (16 h) compared to MLV (Senior and Gregoriadis, 1982).

c) Large unilamellar vesicles

LUV vesicles are vesicles that usually have a diameter range greater than 100 nm. Like SUV, they have a single lamella and lower lipid/aqueous distribution ratio. Therefore they offer optimal loading capacity for water soluble drugs and lower capacity for lipid soluble drugs (Liu, 2000). They are rapidly cleared by Reticulo-endothelial system (RES), even though they have ability to entrap large percentage of hydrophilic drugs and possess a high aqueous space-to-lipid ratio (Szoka and Papahadjopoulos, 1978).

d) Oligolamellar vesicles (OLV)

OLV vesicles are vesicles that have large central aqueous core surrounded by five lipid bilayers. The Oligolamellar vesicles vary in size from 0.1 to 1µm. They are composed of several lamellas (Sherril D. Christian, 1995). In 1978, Szoka and Papahadjopoulos (Szoka and Papahadjopoulos, 1978) used reverse phase separation technique in order to generate oligolamellar vesicles. These carriers are easily attacked by RES. Moreover, preclinical and clinical pharmacology studies showed that oligolamellar vesicles are deficient in the stability required to deliver drug to target organs (Lasic and Papahadjopoulos, 1998). Therefore, these lipid vesicles are not ideal choice for drug delivery.

e) Multivesicular vesicles (MVV)

Multivesicular vesicles are vesicles that have multicompartmental structure and the usual diameter is above 1000 nm (Rongen et al., 1997).

Apart from the size, based on interaction mechanism, liposomes are generally classified into three main categories such as conventional liposomes, sterically stabilised liposomes (stealth liposomes) and polymorphic liposomes (Gregoriadis, 1976).

a) Conventional liposomes are made up of simple lipid bilayers having phosphatidylcholines and cholesterol.

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b) Stealth liposomes or sterically stabilized liposomes consist of lipid bilayers that can be coated with polyethylene glycol. Polyethylene glycol coating not only stabilises the lipid bilayer but also protects the liposomes from macrophages and RES. Consequently, stealth liposomes, compared to conventional liposomes, are retained longer in blood. Basically stealth liposomes are inert type of material and non-reactive towards the surrounding.

c) Polymorphic liposomes are also known as activosome. They are very reactive towards specific agents. External stimuli alter the morphology of these liposomes. Categories such as cationic liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes and target specific liposomes are included into this. For instance cationic liposomes for nucleic acid delivery (Lasic D., 1996). As a delivery system of gene, cationic liposomes have several advantages. Firstly, the positive charged liposomes are easily biodegradable after administration *in vivo*. Secondly, the lipid composition-dependent modulation of surface charge density can regulate the interaction forces with negatively charged nucleic acids (Shim et al., 2013). Additionally, the insertion of lipophilic drugs in the lipid bilayers of cationic liposomes can deliver both anticancer drug and therapeutic nucleic acids (Shim et al., 2011). As a result, cationic liposomes have been considered for the delivery of plasmid DNA (Hofland et al., 1996), Aptamers (Xiong et al., 2011) and siRNA (Barron et al., 1999).

In this way, vesicles bilayer structure and interaction mechanism decide the types of liposomes. However it is important to understand how these different types of liposomes are generated, their characterization techniques and physicochemical attributes of liposomes play crucial role in the formulation and pharmacokinetics of liposomes.

1.2.3. Preparation of liposomes

Since 1965, numerous methods have been employed for formulation of liposomes. Among these, fifteen are most important methods (Lasch et al., 2003; Barenholz and Lichtenberg, 2009). Below is the list of common methods used to generate different types of vesicles (Fig 1.5).

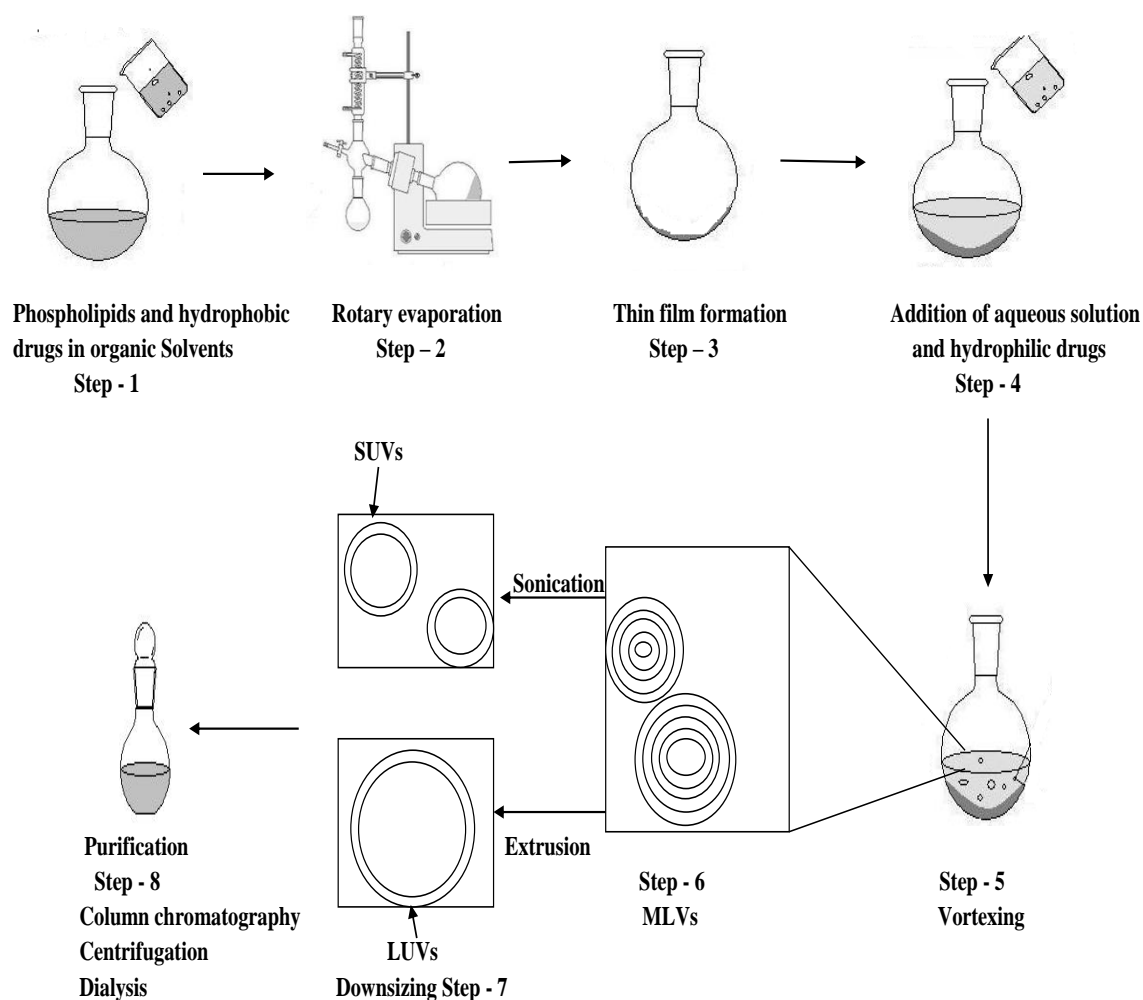


Fig 1.5: Schematic representation of preparation of liposomes via different techniques [adapted from (Lopes et al., 2013)].

a) Thin Film Hydration Method (Bangham Method) (refer to Fig 1.5)

Thin film hydration method, also termed as Bangham method (Bangham et al., 1965), is a simple technique to generate MLV. The method involves the introduction of phospholipids into a mixture of solvents (i.e. chloroform/methanol) that is then evaporated by vacuum. When the solvent is completely removed, the phospholipids are dried on the walls of the round bottom flask as a thin film. This thin film is then hydrated with aqueous phase. Instantly, phospholipids are hydrated and lamella like structure grow into thin lipid tubules (Lasch et al., 2003). Those are very hard to detach from the wall of round bottom flask (RBF). Merely mechanical agitation such as vortexing, swirling, shaking etc. induce the thin lipid tubules to split and repack the hydrophobic edges of lipid tubules into a big spherical liposome i.e. MLVs. As mentioned earlier these big spherical liposomes are composed of five to twenty lipid bilayers (onion like structure) that internally acquire small aqueous phase (O'Doherty, 2004).

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b) Ultrasonication (refer to Fig 1.5)

This method is also termed as ultrasonic irradiation (Barenholz and Lichtenberg, 2009). Traditionally ultrasonication was the first mechanical treatment that involved the sonication of aqueous phosphatidylcholine dispersions (MLV) (Papahadjopoulos and Watkins, 1967). The method involves the formation of MLV by thin film hydration technique as described in thin film hydration method section above. Furthermore, with the help of ultrasonic irradiation above the (phase transition) T_c of phospholipids, opalescent dispersions of phosphatidylcholine (MLV) turned into the clear dispersions of phosphatidylcholine. These clear dispersions of phosphatidylcholine are due to formation of SUV (Barenholz et al., 1977; Huang, 1969). Ultrasonic irradiation is done by two methods –probe sonication and bath sonication (Lasch et al., 2003). In the case of probe sonication, the sonicator tip is directly immersed into the phospholipids dispersions whereas in the bath sonicator, phospholipids dispersions in a tube or beaker are positioned into bath sonicator. Probe sonication is the most commonly used method for the preparations of SUV. This method is very fast and can be directly applied to MLV dispersions because of higher energy output (Johnson et al., 1971; Papahadjopoulos and Watkins, 1967). But probe sonication has limitations such as, SUV dispersions is formed along with contamination of metal ions such as titanium particles that come from tip of probe, and temperature of MLV is not fully controlled due to open system as probe is immersed into the MLV dispersion (Barenholz and Lichtenberg, 2009). SUV that are produced by ultrasonication are usually metastable (Lasch et al., 2003).

c) Extrusion (refer to Fig 1.5)

Large unilamellar vesicles can be prepared by extrusion. Extrusion is a technique in which micrometric liposomes, such as MLV suspensions, are forced through polycarbonate filters (100 nm of pore size) to generate LUV possessing diameters near the pore size of the polycarbonate filters used (Hope et al., 1986; Olson et al., 1979). Preceding extrusion through the polycarbonate filters, MLV suspensions are generally disrupted either via several freeze-thaw cycles or via prefiltering of the MLV suspensions. This process thwarts the lipid membranes from fouling and recovers particle size distribution homogeneity of the final suspension.

d) Freeze-dried rehydration vesicles

With the aim of increasing the entrapment efficiency of preformed vesicles, (Kirby and Gregoriadis, 1984) invented a well-known method called dehydration-rehydration vesicles. These vesicles entrap high amount of macromolecules. Ideal freeze-dried rehydration liposomes having high amount of proteins are produced by joining three methods (Lasch et al., 2003). The initial method in which SUV is successfully produced, is the same as mentioned earlier in ultrasonication section. The second methodology described by Kirby and Gregoriadis (Kirby and Gregoriadis, 1984) in which SUV are mixed together with the proteins solution to be entrapped before freeze

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drying, and then a drying step causes close intact of the lipid bilayers and proteins. During rehydration-dehydration step, SUV become destabilised and by fusion, freeze-dried rehydration liposomes entrapping high amount of proteins are obtained. Last method is extrusion method in which homogenization and size reduction of freeze-dried rehydration vesicles is done by polycarbonate filters.

e) Reverse- phase evaporation techniques

During 1978, Szoka and Papahadjopoulos (Szoka and Papahadjopoulos, 1978) successfully introduced reverse-phase evaporation method and developed reverse phase vesicles (REV) such as LUV and oligolamellar vesicles. These liposomes have the ability to entrap large percentage of hydrophilic drugs and possess a high aqueous space-to-lipid ratio. A range of lipids or lipid mixtures including cholesterol can be used to prepare REV and possess higher aqueous-to-lipid volume ratio as compared to SUV and MLV (Lasch et al., 2003; Smirnov, 1984).

f) Ether and petroleum ether vaporization method

This method is also termed as ether and petroleum ether infusion method (Barenholz and Lichtenberg, 2009). In this method, a single lamella is produced through the injection of phospholipids dissolved in an organic solvent into a large amount of hot water which is heated to 60 °C. Due to high temperature, instant evaporation of organic solvent, i.e. evaporation of an ether or petroleum ether, causes the phospholipids to aggregate and form osmotically active single lamella that has a high aqueous space-to-lipid ratio (Cortesi et al., 1999; Barenholz and Lichtenberg, 2009). In 1972, Chowhan et al. (Chowhan et al., 1972) developed multilamellar liposomes through this technique. Later on, Deamer and Bangham (Deamer and Bangham, 1976) effectively developed osmotically active large volume liposomes. Volume trapping efficiency of these liposomes is nearly ten times more as compared to vesicles obtained through sonication and hand shaken techniques.

g) Detergent depletion

This method involves the removal of detergent and formation of vesicles lamella. Classically four techniques are used for the detergent depletion (Lasch et al., 2003).

- 1) Dilution (Schurtenberger, 1984)
- 2) Dialysis (Milschmann et al., 1978)
- 3) Gel filtration (Brunner et al., 1976)
- 4) Adsorption (Holloway, 1973)

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The size of liposomes that are obtained through detergent depletion techniques fully depends on the types of phospholipids and detergent. On lesser extent it depends on the method employed for the detergent depletion (Barenholz and Lichtenberg, 2009). This technique is extremely flexible. This facilitates the formation of various liposomes and recombinant proteoliposomes (Ollivon et al., 2000). Most of the techniques like extrusion and sonication can sometime physically affect the functional properties of proteins and enzymes. On the safer side detergent depletion is very gentle and retains the nature of sensitive proteins and enzymes. Sodium cholate and alkoxypolyethylenes are most popular detergents that are frequently used in the detergent depletion technique (Matz and Jonas, 1982).

h) High pressure homogenization

High pressure homogenization is achieved by different types of homogenizers. This method involves the passage of liposomes suspension through a sequence of nozzles of homogenizers (Barenholz and Lichtenberg, 2009). Due to high shear of nozzles, liposomes suspension is broken down into the desired liposomes. As per the size requirement of liposomes, nozzles size can be attuned. Homogenizers that are used for liposomes preparation are mainly categorised into three types (Lasch et al., 2003).

- 1) The APV homogenizer
- 2) Microfluidizer
- 3) Ultra-Turrax homogenizer

Practically Homogenizers are important tool and are utilised for the liposome preparation in three common ways.

1. In the first place liposomes are produced and then passed into the homogenizer to obtain the desired size of lamella. e.g. multilamellar vesicles (Mayhew et al., 1985) or dehydrated-rehydrated vesicles (Gregoriadis, 1990).
2. Production of excess amount of SUV (Brandl et al., 1990).
3. Ether evaporation method as mentioned earlier homogenizers are used to homogenize the preparation of phospholipids dissolved in organic solvent that are later injected into the warm water.

i) Interdigitation fusion method

Production of LUV via interdigitation fusion method was introduced by (Ahl et al., 1994). The method involves the addition of ethanol into the SUV which is further heated above the main transition temperature of vesicles. All these conditions cause bilayer interdigitation and LUV is generated. The phase transition temperature of vesicle (T_c or T_m), i.e. the temperature that is

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essential for inducing the vesicle melting from a solid-ordered (gel) to a liquid-disordered (fluid) phase. The phase transition temperature of liposomes can be easily measured by calorimetry (Enders et al., 2004), magnetic resonance spectroscopy (YashRoy, 1990) and surface tension measurement (Gugliotti et al., 1998). Interdigitated fusion method is a very simple technique and the produced LUV have the ability to entrap large percentage of hydrophilic drugs (Smith et al., 2007). One of the main objectives of this research is to prepare photosensitive LUV by interdigitated fusion method.

Apart from these methods, methods such as calcium induced fusion in order to prepare LUV by EDTA (Papahadjopoulos et al., 1975); production of LUV by using nonelectrolyte solution (Reeves and Dowben, 1969); lyophilization or freeze drying techniques for the production of MLV (Szoka and Papahadjopoulos, 1980) are also used for the preparation of liposomes. Following the liposome preparation, it is also essential to characterize these vesicles. Characterization of liposomes provides information on the stability of the formed vesicles.

1.2.4. Characterization of liposomes

Different methods are used to characterize liposomes and they are classified as physical characterization and chemical analysis of liposomal components (Zuidam, 2003).

1.2.4.1. Physical characterization

Physical characterization of liposomes includes parameters such as the vesicles size distribution, zeta potential, lamellarity determination and entrapment efficiency of liposomes.

a. Vesicle size distribution

The size and size distribution of vesicles are very important and play a key role in parental drug delivery (Guiot et al., 1980; Vemuri et al., 1990). Liposome stability entirely depends on size and size distribution of vesicles. Several methods are used for the determination of vesicles size. One of the well-known approaches is electron microscopy. Electron microscopy such as freeze fracture microscopy is a precise method that allows one to view each individual vesicle. Visual inspection of liposomes is becoming easier with this techniques (Hope et al., 1985), but it is time-consuming. Another method which comes into this category is laser light scattering analysis. Liposomes up to 1 μm can easily be determined by this method with instruments such as Mastersizer™, Sympatec GMBH Zetasizer (Germany) etc. The technique is dependent upon the dynamic scattering phenomena. In theory particles are suspended into a solution and then illuminated by a laser light.

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As the index of refraction of particles is different from the index of refraction of suspending medium, particles scatter laser light and give signal to noise ratio (Bezot et al., 1978; Cummins and Pike, 1974). The limitation associated with this technique is insufficiency in determining aggregates of more than one particles. It considers them as single particle. Photon correlation spectroscopy also comes into this category. This is useful for the determination of small vesicles and is based on the Brownian motion of suspended particles (Ostrowsky, 1993). All these methods require expensive equipment. Last not least method that falls into this category is gel exclusion chromatography (Barenholz and Amselem, 1993; Lesieur et al., 1993; Sommerville and Scheer, 1987). The requirement of this technique is gel materials and buffer.

b. Zeta potential

Another important factor which characterizes the liposomes is zeta potential. Lipid vesicles in solution always carry an electrical charge depending on the type of lipid composition (cationic lipid, anionic lipid and neutral lipid). This is termed as zeta potential and it is determined by measuring the velocity of particles (Cevc, 1993), e.g. ZetasizerNano ZS (Malvern,UK), Zetaplus (Brookaven,USA) etc. It also determines strength of attractive force and repulsive forces within the particles. In general zeta potential plays crucial role in liposome stability and drug delivery (Mozafari, 2009).

c. Lamellarity determination

As mentioned earlier in liposomes classification, lamellarity of liposomes can determine the type of liposomes. This lamellarity formation depends on the type of lipid and the method of preparation (Edwards and Baemner, 2006). Various techniques such as derivatization of the outer surface of bilayer; Nuclear magnetic resonance (NMR), Small-angle X-ray scattering (SAXS) and electron microscopy are used for the lamellarity determination (Zuidam, 2003). The derivation of the outer surface of bilayer is achieved through the combination of chemical method with spectroscopy. Procedures involve the production of trinitrophenylated (TNP)-phosphatidylethanolamine (PE) and measurement of this product by spectroscopy (Barenholz et al., 1977). Other technique used for lamellarity determination is nuclear magnetic resonance (NMR). In this, lamellarity determination is fully based on the addition of positively charged manganese ions (Mn^{2+}) into the liposomes (Hope et al., 1985). In theory, liposomes are made up of phospholipids. All phospholipids head group contains phosphates that carry negative charge. When the manganese ions are added into the liposomes, they react with phosphorous which is situated on the outer surface of lamella and give broadened peak. The phospholipids head group which is located inside the vesicles does not react with manganese and give normal phosphate peak. Comparison between the broadened and normal peaks can easily determine the liposome lamellarity. A serious limitation of this technique in case of negatively charged liposomes is that positively charged manganese ions can provoke the fusion of

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liposomes and give false results. Lamellarity is also characterized by electron microscopy (Sommerville and Scheer, 1987) and small angle X-ray scattering (SAXS) (Bouwstra et al., 1993). SAXS allows the determination of the structure of individual vesicles aggregates as well as the structure of vesicular dispersions (Petoukhov and Svergun, 2013). SAXS also measure the membrane thickness and the bilayer–bilayer separation. The basic principle behind SAXS analysis is to scatter X-ray photons elastically off molecules in solution and to record the scattering intensity as a function of the scattering angle (Schmidt, 1971). This technique is very ideal, as this is non-invasive and allow the characterisation of complex surfactant fluids in their natural state (Blanchet and Svergun, 2013).

d. Entrapment efficiency of liposomes

Entrapment efficiency of liposomes is commonly measured by entrapping water soluble markers such as radiolabel markers ($[^{14}\text{C}]$ glucose, $[^{14}\text{C}]$ Inulin, $[^{99\text{m}}\text{Tc}]$ DTPA); enzymatic markers (soybean trypsin inhibitor, Superoxide dismutase) and fluorescent markers (Fluorescein, Calcein)(Zuidam, 2003). The detection techniques which are used for the quantification of these hydrophilic markers are spectroscopy (NMR, electron spin resonance (ESR)), fluorometry, enzyme based assay, chromatography (gel exclusion chromatography, column chromatography, ion exchange chromatography), ultracentrifugation and electrochemical methods (Jesorka and Orwar, 2008; Madden et al., 1990). The mechanism behind the determination of entrapment efficiency of liposomes is very simple and straightforward. The liposome formulation contains both untrapped and trapped drug materials (Vemuri and Rhodes, 1994b; Vemuri and Rhodes, 1994a; Vemuri and Rhodes, 1995; Vemuri et al., 1990). The untrapped drug materials are also termed as free drug materials. Usually these untrapped drug materials are separated from the trapped drug materials by ultracentrifugation. Then the trapped materials are treated with lysis agent such as Triton x-100 and isopropanol. On the addition of these lysis agents, the fraction of drug materials released from the vesicles into the solvent is then quantified by spectroscopy and chromatography (Vemuri and Rhodes, 1994b).

1.2.4.2. Chemical analysis of phospholipids

By using chromatography and chemical assays, the phospholipids are easily characterized (Chatterjee and Banerjee, 2002). The table 1.1 illustrates the techniques used for the chemical analysis of phospholipids (Zuidam, 2003).

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Table 1.1: Characterization techniques for the determination of phospholipids

	Chemical stability	Techniques
1.	Hydrolysis of phospholipids	HPLC, HPTLC
2.	Determination of amount of non- esterified fatty acids	enzymatic assay, HPLC
3.	Autoxidation of phospholipid acyl chain	Conjugated dienes, lipid peroxides,
4.	Autoxidation of cholesterol	TLC, HPLC
5.	Degradation of active compounds	TLC, HPLC
6.	Degradation of antioxidant	TLC, HPLC

In this fashion liposomes are prepared and characterized. But apart from the method of preparation, the study of another parameter i.e. physicochemical attributes of vesicles are imperative.

1.2.5. Physicochemical attributes of vesicles

Modifications in physicochemical attributes of vesicles not only influence the liposome formulation and stability, but also the drug encapsulation and pharmacokinetic behaviour of liposomes *in vivo*. This involves factors such as vesicles size and lamellarity, surface charge, dose of lipid, vesicles composition and structure, steric stabilization (Drummond et al., 1999; Lasic et al., 1992; Sharma and Sharma, 1997).

a) Vesicle size and lamellarity

The first influential factor which alters the pharmacokinetics of liposomes *in vivo* and drug permeability is vesicle size and lamellarity (Drummond et al., 1999; Yamauchi et al., 2007). The liposomes which are prepared by extrusion technique contain defined size ranging from 0.1 to 0.2 μm (Szoka et al., 1980). But the actual diameters of these liposomes prepared through extrusion are always greater (20-50 %) than the pore size of nanoporous filters. Due to this increment these large vesicles are easily cleared out by RES through opsonisation (Hwang, 1987; Senior, 1987). This work is also supported by (Klibanov et al., 1991), and they proved that liposomes with diameters higher than 200 nm are easily cleared by RES as compared to the liposomes with diameters less than 100 nm. The rate of clearance also depends on the lipid composition. In most cases the lipid geometry can increase the size. For instance MLV containing phosphatidylcholine, cholesterol and dicetyl phosphate in the ratio of (7:2:1) are rapidly cleared out by RES (Gregoriadis and Ryman, 1972). In diseases like cancer, liposomes containing vesicles size between 100 nm and 200 nm are easily permitted into the tumour tissue as compared to the liposomes containing vesicle size greater

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than 200 nm (Mayer et al., 1989; Papahadjopoulos et al., 1991). In addition, liposome size and lamellarity also influence the drug permeability. It has been shown that larger vesicles retain vincristine for longer period with slow the release (Yamauchi et al., 2007). Following NMR and trap volume measurement they have concluded that larger vesicles have greater size that ultimately gives the rigidity to the liposomes and slow the permeability of drug.

b) Surface charge

The second aspect of liposomes that amend the liposome stability, pharmacokinetics of liposomes *in vivo* and vesicles-cell interaction is the surface charge of liposomes (Drummond et al., 1999; Sharma et al., 1993b; Sharma and Straubinger, 1994). Liposomes are made from cationic lipids, anionic lipids and charge free lipids. Among these the liposomes which are made from charge free lipids are unstable and aggregate easily. For example, charge free lipids in case of SUV lead to aggregation due to their low surface potential (Sharma and Straubinger, 1994) whereas liposomes which are made up from cationic and anionic lipids do not form any aggregates. The reason behind this is the formation of electrical double layer in these formulations. As per DLVO, electrical double theory, similar charged lipid particles repel each other and avoid fusion (Russel, 1989). Numerous investigations have proved that liposomes resultant from the anionic lipids such as phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidic acid (PA) are easily cleared by mononuclear phagocyte system (MPS) (Drummond et al., 1999; Senior, 1987). A detailed study on the clearance of charged liposomes has been done on mice by (Gabizon and Papahadjopoulos, 1992). In this study liposomes containing anionic lipid were cleared by MPS in the following order (Phosphatidylserine>phosphatidic acid >phosphatidylglycerol). But the incorporation of another sterically shielded negatively charged lipid i.e. PEG-PG conjugate increased their half-life ($t^{1/2}$) (Drummond et al., 1999; Papahadjopoulos et al., 1991). Liposomes containing neutral lipids are not easily cleared by MPS (Sharma et al., 1993b), in spite of the fact that they avoid the significant reaction with the cells. Therefore it is difficult to deliver drugs through these types of liposomes and only after leakage they can enter into the cell (Sharma and Sharma, 1997). Liposomes containing negatively charged lipids easily react with the cells / blood components and by endocytosis mechanism they release their contents into the blood (Sharma et al., 1993a). Liposomes resulting from cationic lipids significantly interact with cell membrane and through liposome-cell fusion mechanism they can deliver their contents into the cell (Felgner et al., 1994). Cationic lipids are usually used for gene delivery because of the polyanionic nature of DNA (Balazs and Godbey, 2011; Hofland et al., 1996). Briefly, Cationic lipids commonly acquire a positive charge through one or more amines that are present in the polar head region. This positively charge facilitates electrostatic binding with anions such as those found in DNA (Ruponen et al., 1999).

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c) Dose of lipid

The administered dose of lipid influences the pharmacokinetic behaviour of liposomes (Drummond et al., 1999). Half-life of conventional liposomes can be increased by a dose dependent process. Numerous experiments have established that an increment in the dose of liposomes reduces their plasma opsonisation and increases their half-life indicating saturation mechanism liable for their RES uptake or non-linear pharmacokinetics (Abra and Hunt, 1981; Drummond et al., 1999; Senior et al., 1985). In contrast, sterically stabilised liposomes such as 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (DSPE-PEG) are potential for liposomal drug delivery because their linear pharmacokinetics of plasma clearance is independent of dose and half-life remains unaffected with larger dose (Allen and Hansen, 1991; Beaumier et al., 1983; Drummond et al., 1999).

d) Vesicles composition and structure

Apart from the vesicle size and surface charge, vesicle bilayer composition and choice of phospholipids play major part in manufacturing, stability and *in vivo* performance of liposomes. Liposomes are made up of phospholipids. The therapeutic activity of liposomes depends on the type of phospholipids. Five different types of phospholipids are available so far and are used to make liposomes (Barenholz and Crommelin, 1994; Kriftner, 1992; O'Doherty, 2004). These are outline as follows:

1. Phospholipids derived from natural sources- Phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sphingomyelin (SPM) fall into this category and they are obtained from two natural sources; egg and soya. Structurally, they are mixture of acyl ester phospholipids and unsaturated acyl chains, retaining double bond in the *cis* form. Acyl chain position can distinguish phospholipids from egg and soya (Lichtenberg and Barenholz, 1988).
2. Phospholipids modified from natural sources- these are the phospholipids that are readily obtained by performing modification in already existed natural phospholipids. Modification involves partial or full hydrogenation of the natural phospholipids; and resultant phospholipids possess high transition temperature with shorter unsaturated structure .e.g translation of phosphatidylcholine into phosphatidylethanolamine (Eible, 1981).
3. Semi-synthetic phospholipids- these are commercially obtained after replacing the acyl chain assembly of natural phospholipids with defined acyl chain (O'Doherty, 2004) e.g. conversion of phosphatidylcholine to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

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4. Fully-synthetic phospholipids- these types of phospholipids are generally prepared by synthesis e.g. erucylphosphocholine (ErPC) is a more effective drug than hexadecylphospholine (HePC) for systemic intravenous treatment of cancer (Eibl and KaufmannKolle, 1995). This is due to the fact that ErPC have a longer 22 carbon chain with a ω -9-cis-double bond as compare to HePC (Berger et al., 1998). This structural alteration resulted in reduced hemolytic activity, thereby facilitating intravenous administration (Kaufmann-Kolle et al., 1996). Furthermore, the erucylphosphocholine (ErPC) effectiveness can also be explained by the fact that ErPC generate preferentially lamellar structures in aqueous solutions instead of micelles (van Blitterswijk and Verheij, 2013). Fully-synthetic phospholipids offer many advantages including that the composition of fatty acids remains unchanged. In addition, the phase transition temperature and bilayer fluidity of phospholipids are easily adjusted by selecting proper acyl chain and degree of saturation. But the preparation of fully synthetic phospholipid is very expensive and that is the limitation.

5. Phospholipid with non-natural headgroups – in this approach, the head groups of phospholipids are derivatised with other agent. So far in case of immunoassays, phospholipids with non-natural headgroups such as avidin and streptavidin are used as generic liposome labels. For instance biotin conjugated lipids such B-PE can be built on liposome membrane (Rongen et al., 1997).

Selections of proper phospholipids are essential. It not only plays a key role in case of bilayer rigidity but also to release of entrapped drug materials from the liposomes. For instance, fully synthetic phospholipid molecule, i.e. erucylphosphocholine (ErPC), is very effective for cancer treatment compared to hexadecylphospholine (HePC) (Eibl and KaufmannKolle, 1995). Phospholipids are lipids and they all possess special thermal characteristics including a phase transition temperature (T_c), and the temperature below and above the phase transition temperature (T_c) can decide their physical states such as gel like phase (solid form) exist below the (T_c) and sol like phase (liquid crystalline form) exist above the (T_c) (Deb, 2001). This phase behaviour can significantly affect the drug release mechanism from liposomes as well. By elevating the temperature of liposome preparation, the gel form of phospholipid bilayer melts and is transformed into the liquid form and that leads the release of entrapped drug materials (Fig 1.6) (RRC, 1990).

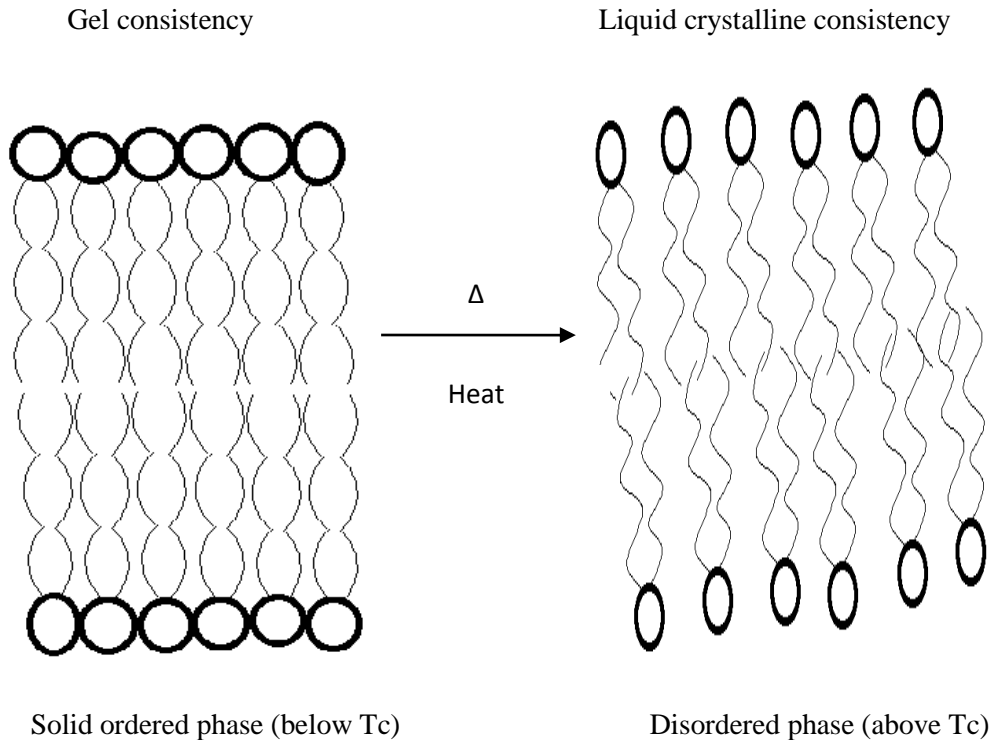


Fig 1.6: The phase behaviour of lipid bilayer with increased temperature.

The phase transition temperature (T_c) of a phospholipid bilayer depends on four variables:
(Mozafari, 2009)

- I. Degree of saturation of the hydrocarbon chains
- II. Polar head group.
- III. Length of acyl chains
- IV. Strength and ionic nature of the suspension medium.

Therefore, in theory, the phase temperature (T_c) of liposomes can be lowered by decreasing the length of acyl chains, unsaturation of the acyl chains and the presence of bulky polar head groups (Szoka and Papahadjopoulos, 1980). Furthermore, T_c can fluctuate between 15 °C for egg yolk phosphatidylcholine equipped with high degree of unsaturation of the hydrocarbon chains to above 50 °C for fully saturated distearoylphosphatidylcholine (DSPC) (Barenholz and Crommelin, 1994; O'Doherty, 2004). Phase transition temperature is essential in the case of liposome formulation and that is why liposomes are not obtained at temperatures below the phase temperature of phospholipids. With cholesterol addition, it is decreased to some extent, but not excluded (Leserman et al., 1994). Most of the experiments have also suggested liposome formulation to be conducted at temperatures well above the phase transition temperature of phospholipids. For

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example the phase temperature of dipalmitoylphosphatidylcholine (DPPC) is 41 °C and it has been recommended that liposomes formulation will be conducted at 10 °C greater than the phase temperature of DPPC i.e. 51 °C (Jurimaromet et al., 1990; Jurimaromet et al., 1992). A study of phase transitions and fluidity of phospholipid membranes is very important as they affect phospholipid-cell interaction *in vivo*. Liposomes which are composed of higher (T_c) phospholipids appear to have lower rate of clearance by RES as compared to liposomes possessing low (T_c) phospholipids (Gabizon and Papahadjopoulos, 1988). However inclusion of steroid molecule, i.e. cholesterol, into the bilayer of membrane can significantly maintain the bilayer stability and increase half-life of liposomes *in vivo* (Drummond et al., 1999; Kirby et al., 1980; Senior and Gregoriadis, 1982; Senior, 1987).

e) Steric stabilization-

Steric stabilization is the process in which liposomes are coated with a small fraction (5-10 mole %) of hydrophilic materials such as monosialoganglioside (GM₁), phosphatidylinositol (PI) and polyethylene glycol conjugated with phospholipid (PEG-DSPE). This process of inclusion of hydrophilic materials in liposome preparation makes liposomes sterically stabilised and hence prevent the liposomes interacting with cell and blood proteins (Allen and Chonn, 1987; Allen et al., 1985; Gabizon and Papahadjopoulos, 1988; Papahadjopoulos et al., 1991). As these liposomes avoid the interaction with cells and blood proteins, they will not be cleared by RES and are therefore considered as long circulating liposomes (Allen et al., 1985; Needham et al., 1992; Papahadjopoulos et al., 1991). One of the great advantages of these sterically stabilised liposomes is their unsaturability and linear pharmacokinetics (Allen and Hansen, 1991; Beaumier et al., 1983; Drummond et al., 1999).

1.2.6. Applications of liposomes in drug delivery

From the drug delivery point of view, Liposomes are one of the safest nanocontainer when compared to other nanomaterials (e.g. quantum dots, nanowires etc.). Thus, it is widely used in several drug delivery system such as gene (Barron et al., 1999) and cancer drug delivery (Priebe and Perez-Soler, 1993), skin drug delivery and other clinical purpose (Samad et al., 2007).

1.2.6.1. General considerations of clinically approved and emerging liposomes

Liposomes have been studied for a long time (Allen and Hansen, 1991; Beaumier et al., 1983; Drummond et al., 1999; Fenske et al., 2008; Fenske and Cullis, 2008). Following these studies, liposomes containing neutral and negatively or positively charged phospholipids along with cholesterol have been successfully prepared. A Few of these formulations have been approved for

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use in humans and others have reached Phase I/II/ III clinical trials. Table 1.2 (page 42) illustrates some examples of clinically approved liposomes and emerging liposomes for therapeutic use (Villaverde, 2011; Elbayoumi and Torchilin, 2009). It is remarkable to mention that DaunoXome (liposomes containing anticancer drug Doxorubicin Nexster pharmaceuticals) and Doxil/Caylex (PEG-liposomes containing anticancer drug Doxorubicin, Sequus pharmaceuticals) were the two honourable liposome based formulations approved for patients in clinics (Allen and Martin, 2004; Batist et al., 2001; Davies et al., 2008).

Other primary examples of clinically approved liposome based products are AmBisome (Gilead Sciences, FosterCity, USA) which is used to deliver the antifungal drug amphotericin B (Veerareddy and Vobalaboina, 2004), Myocet (Elan Pharmaceuticals, USA) which is used to deliver anticancer drug doxorubicin (Alberts et al., 2004). Currently DaunoXome is considered as a stable liposomal formulation as this is not cleared by MPS opsonization and available in ready- to-inject formulation (Immordino et al., 2006).

A number of formulations have also been prepared by combining sphingomyelin phospholipids and conventional liposomes (Immordino et al., 2006). For instance novel liposomal formulation vincristine (Marqibo Hana biosciences, USA) has been approved as orphan medical products in US and Europe and used to treat uveal melanoma (Bedikian et al., 1995). Furthermore, liposomal formulations such as INX-0125 (liposomal vinorelbine) (Semple et al., 2005) and INX-0076 (topotecan) (Tardi et al., 2000) are also used as potential therapeutic agent. Clinical data have shown that incorporation of OSI-211 in the acid aqueous core of the vesicle can increase stability of the active compound as well as improve its tumour toxicity (Seiden et al., 2004). Therefore, OSI-211 (Lurtotecan, OSI Pharmaceuticals, Inc., NY) has been successfully used to treat ovarian cancers and B-cell lymphoma (Duffaud et al., 2004; Lu et al., 2005).

A large number of MLVs from Neo Pharma Inc. have been used clinically and these include liposome formulations such as LEM-ETU (mitoxantrone-entrapped) (Ugwu et al., 2005), LEP-ETU (paclitaxel-loaded) (Yavlovich et al., 2009; Zhang et al., 2005) and LESN38 (irinotecan-encapsulated) (Lei et al., 2004; Pal et al., 2005). Recently, DepoCyt (Pacira Pharmaceuticals, San Diego, CA)- a liposomal formulation entrapping cytarabin has been approved for neoplastic meningitis and lymphomatous meningitis and given by intrathecal route (Glantz et al., 1999; Jaekle et al., 2001; Mantripragada, 2002; Phuphanich et al., 2007). Other two MLV liposome formulations possessing saturated phospholipids such as dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) have been approved for clinical use. For instance Nyotran® (Aronex Pharmaceuticals, The Woodlands, TX, USA) for treating antifungal infection (Anonymous (1999); Arkan and Rex, 2001) and Aroplatin® (Antigenics Inc., Lexington,

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MA, USA) for treating B-cell lymphoma (Han et al., 1993; Lu et al., 2005; Verschraegen et al., 2003). Another remarkable liposomal formulations is made by Aronex Pharmaceuticals named as Atragen® which contains the active ingredients tretinoin, dimyristoylphosphatidylcholine (DMPC), soybean oil and is very effective against retinoid-responsive cancers and acute promyelocytic leukemia (Ozpolat et al., 2003).

A lipophilic anthracycline antibiotic i.e. Annamycin liposomes is primarily investigated by MD Anderson Cancer Institute (Priebe and Perez-Soler, 1993). This formulation has reached Phase II clinical trials and is being investigated as potential tool in the treatment of doxorubicin resistant breast cancer (Booser et al., 2002). Another potential drug candidate which entered Phase II clinical trial is EndoTAG™-1 (MediGene A.G., Martinsried, Germany). This is effective against pancreatic cancers (Eichhorn et al., 2006). Apart from this, Visudyne® was approved by FDA in 2000 and for the first time age related macular degeneration was successfully treated with these liposomes. Other clinically approved liposomes are DepoDur® (containing morphine) which is used for postoperative pain after surgery; Definity® (containing octafluoropropane) for ultrasound imaging; Abelcet® (containing amphotericin- B) for fungal infection and Mepact® (containing Muramyl dipeptide) for osteosarcoma. During 2009, Octocogalfa (containing plasma factor VIII) has been approved for the treatment of haemophilia A.

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Table 1. 2: Clinically approved liposomes and emerging liposomes for therapeutic use
[adapted from (Allen and Cullis, 2013)]

Marketed product	Company and marketing name	composition	API	Target diseases	Trial status
DaunoXome®	Nexstar Pharmaceuticals, 1995	DSPC/CHOL	Daunorubicin	Kaposi's sarcoma	approved
DOXIL®/Caelyx®	Sequus Pharmaceuticals, 1997	SoyHPC/CHOL/DSPE-PEG	Doxorubicin	Kaposi's sarcoma	Approved
Myocet®/Evacet®	ElanPharma, 2000	EPC/CHOL	Doxorubicin	Metastatic breast cancer	Approved
SPI-077	Sequus Pharmaceuticals	SoyHPC/CHOL/DSPE-PEG	Cisplatin	Head and neck cancer	I/II
Lipoplatin™	Regulon Inc.	SoyPC/DPPG/CHOL	Cisplatin	Several cancer type	II/III
Aroplatin	AntigenicsInc	DMPC/DMPG	Oxaliplatin analog	Colorectal cancer	Phase II
Depocyt	SkyePharma 1999	DOPC/DPPG/CHOL/triolein	Cytarabin	Lymphoma meningitis	Approved
LEP-ETU	NeoPharmInc	DOPE/CHOL/cardiolipin	Paclitaxel	ovarian, breast and lung cancer	Phase I

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Marketed product	Company and marketing name	composition	API	Target diseases	Trial status
LEM-ETU	NeoPharmInc	DOPE/CHOL/cardiolipin	Mitoxantrone	leukemia, breast cancer	Phase I
LE-SN38	NeoPharmInc	DOPE/CHOL/cardiolipin	Irinotecan	advanced cancer	Phase I
MBT-0206	MediGene AG	DOPE/DO-trimethylammoniumpropane	Paclitaxel	Breast cancer	Phase I
OSI-211	Enzon Co.	SoyHPC/CHOL	Lurtotecan	Ovarian cancer Head and neck cancer	Phase II
Marqibo®	Inex Pharm	DSPPC/CHOL/sphingosine	vincristine	Non-Hodgkin's lymphoma	Phase II/III
Atragen®	Aronex Pharm	DMPC, and soybean oil	t-retinoic acid	renal cell cancer,	Phase I/II
INX-0125	Inex Pharm	DSPPC/CHOL/sphingosine	vinorelbine	breast, colon and lung cancer	Preclinical Phase I
INX-0076	Inex Pharm	DSPPC/CHOL/sphingosine	topotecan	advanced cancer	Preclinical
Liposomal-Annamycin®	MD Anderson Cancer centre	DSPC/DSPG/Tween	Annamycin	breast cancer	Phase II
Ambisome®	Fujisawa USA Inc. and Nexstar Pharm 1997	SoyHPC/DSPPC/CHOL	amphotericin	Systemic fungal infections.	Approved
Nyotran®	Aronex Pharm	DMPC/DMPG/CHOL	Nystatin	Fungal infections	Phase II/III
Visudyne®	Novartis,2000	EPG/DMPC	Verteporfin	Age related macular	Approved

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Marketed product	Company and marketing name	composition	API	Target diseases	Trial status
			(BPD-MA)	degeneration	
DepoDur®	Endo pharmaceuticals, 2004	DOPC/Cholesterol/DPPG	Morphine	Postoperative pain after surgery	Approved
Definity®	Dupont Merck, 2001	Gd-DTPA	Octafluoro-propane	Ultrasound imaging	Approved
Abelcet®	Sigma-Tau pharmaceuticals, 2009	DMPC/DMPG/CHOL	Amphotericin -B	Fungal infection	Approved
Mepact®	Takeda, 2009	POPC/OOPS	MTP	osteosarcoma	Approved
Octocogalfa®	Bayer schering ,2009	Octogonalfa/POPC/MPEG20 0-DSPE	factor VIII	Haemophilia A	Approved

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In conclusion, to date a few number of liposome based formulations are clinically approved while other liposome based formulations are in the queue awaiting clinical trials, even though patient treatment can be improved by considering two aspects i.e. drug targeting potential and triggering mechanism of liposomes (Yavlovich, 2010) .

1.2.7. Drug targeting potential of liposomes

The requirement for drug targeting emerges when drug enters into blood after intravenous injection, then most of the drugs is wasted by distribution to other tissues and organs. A small amount of drug reaches to the disease affected area and remaining of the drug causes drug toxicity (Fielding, 1991). In order to increase the availability of drugs to the disease affected area and to diminish the undesirable side effects of drugs, drug targeting through liposomes is essential (Hwang, 1987; Vadie et al., 1989) .

Drug targeting through liposomes has successfully been achieved by the use of a variety of ligands such as antibodies, hormones, peptides and tagged antibodies. This tagged ligand easily determines the exact receptor site and this mechanism pushes liposomes to saturate at such target sites (Torchilin, 1985). In this way, more drugs become available to the target site and distribution of liposomes into the RES region is minimised or precluded. The bio- distribution of lipid vesicles into RES region is effectively altered by the inclusion of protein or carbohydrates molecule (which show specific affinity towards a particular tissue or organ) into the lipid bilayer (Al-Angary et al., 1995; Wolff and Gregoriadis, 1984). Drug targeting via liposomes is very important tool. However, from clinical point of view, success stories from these types of liposomes formulations are less frequent (Yavlovich, 2010). Therefore, it is imperative to focus on another aspect of drug delivery i.e. triggering of liposomes.

1.2.8. Triggering of liposomes

In a bid to increase the availability and diminish the rate of clearance of liposomes, a concept of sterically-stabilized liposomes has come forward. For instance-polyethylene glycol conjugated to distearoylphosphatidylethanolamine (PEG-DSPE). Liposome formulation containing sterically stabilized liposomes exhibit a prolonged circulation time in blood and reduced uptake by the liver and spleen and improved accumulation in implanted tumours (Gabizon and Papahadjopoulos, 1988). Even though, the therapeutic window of these liposomes is very low due to the slow release of drug from the liposomes (Shum et al., 2001). To overcome this drawback, “intelligent” liposomes, which possess energy –activated release mechanism that speeds up the drug release rates and increases the therapeutic efficacy, have been developed (Lehner et al., 2012). These

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“intelligent” liposomes not only improve the efficacy of drug but also increase intracellular stability and activity of drug (Kocer, 2007). Based on the type of stimulus, the “intelligent” liposomes, also termed as functional liposomes, are classified into internally and externally controllable vesicles (Lehner et al., 2012). Internal stimuli are caused by internal triggers such as activation by pH, redox potential and enzymes whereas external stimuli are caused by external triggers such as light, heat, ultrasound, electromagnetic fields or ionizing radiation (Yavlovich, 2010). All triggerable liposomes follow the same mechanism and drug release depends on structural alteration caused by the disruption of the lipid bilayer via a stimulus at the weakest point (Bisby et al., 1999b; Cheong et al., 2006; Ganta et al., 2008; Jeong et al., 2002). Internal triggers possess some limitations. The pH-sensitive liposomes are easily cleared out by receptor mediated endocytosis and that is why they need to be coated with PEGylation material (Gabizon, 2001; Hatakeyama, 2007; Ichikawa et al., 2004). Moreover, all internal triggers (pH, redox potential and enzymes) are difficult to control because the reaction occurs in complex biological systems like an entire organism (Ganta et al., 2008). It is very hard to find the disease specific internal triggers for certain diseases. As a result external triggers are more beneficial than internal triggers for cancer treatment. External triggers can at time focus on certain body areas. So if the one site of target cell is involved in pathogenesis (for instance- stem cells from tumour) then other site of the target cell can easily get targeted by external triggers (for instance –stem cells from bone marrow) (Yavlovich, 2010). External triggers also possess several limitations. Temperature sensitive liposomes cause hyperthermia along with major inflammation (Kocer, 2007). Ultrasound triggered release causes tissue damage whereas electromagnetically and magnetically controllable liposomes require smart design and neutron capturing principles from the tissue which is very difficult (Lehner et al., 2012).

Among all of the triggers, light triggered release is versatile and has distinctive advantages over other systems for the controlled drug release. The major drawback associated with light is tissue penetration depth which could be solved by use of near infra-red (NIR) irradiation. A large number of light triggered liposomes have been approved for clinical trials (e.g. Visudyne). Therefore, one of the objective of this research has been to synthesize photosensitive liposomes which are explained in detail in the following chapter -2.

1.3. Photo-Sensitive Liposomes: Background

Photosensitive liposomes are triggerable liposomes which release their contents on demand upon irradiation of light with an appropriate wavelength (Güven 2013). Biocompatible light-sensitive materials have been scrutinized from the early 1980s (Yavlovich, 2010). These molecular devices that are used for designing liposomes possess the ability to release their load in response to exposure to light (Alvarez-Lorenzo and Concheiro, 2008; Lohmann and Petrak, 1989). The depth of light penetration into the human body depends on the wavelength of light. The ultraviolet (UV) and the blue part of the visible spectrum superficially penetrate into the human body not more than 1-2 mm and allow controlling some processes on the surface and inside the skin. A remarkable increase in penetration begins from orange light (>590 nm). Light with wavelengths exceeding 700 nm (red and near infrared light) penetrate to approximately 1 cm and can be used for controlling light sensitive devices inside the body and also for visualization of some internal processes (Alvarez-Lorenzo et al., 2009; Mochizuki-Oda et al., 2002). Light sensitive lipid molecules have provided an opportunity to create stable nanosystems for controlled release of drugs in circulation together with photo-triggers for localized drug delivery.

In theory, light-sensitive liposomes equipped with synthetic lipids and chromophores are responsible for light harvesting and light-dependent conformational transitions of molecules. In response to the light exposure, derivatives of azobenzene, cinnamoyl, and spirobenzopyran moieties can specifically alter their polarity and corresponding hydrophobicity due to cis-trans isomerization or modification in a number of charged groups (Lohmann and Petrak, 1989; Zhao, 2007; Zhao, 2009). The cis-trans isomerisation of azobenzene can also transform the packing and phase state of lipids. For example, UV treatment of photo-sensitive liposomes containing phospholipid 1, 2-bis [4-4(4-n-butylphenylazo) phenylbutyroyl] phosphatidylcholine (Bis-Azo PC) can initiate transition from a planer structure present in the bilayer vesicles to inverted micellar structures. The conformational transition is reversible and can be controlled by light of different wavelengths. This allows regulation of the permeability and release of internal content from photo-sensitive liposomes containing 6 % of Bis azo PC (Bisby et al., 2000a; Bisby et al., 2000b; Mochizuki-Oda et al., 2002).

Azobenzene derivatives of cholesterol can also be utilized for regulation of liposomes permeability and stability. Cholesterol derivatives with a range of polarity and charge can adjust the sensitivity of liposomes to light exposure and the rate of drug release at different wavelengths and temperatures (Bisby et al., 2000a). The process of isomerization of lipid can also alter the shape of liposomes. For instance, reversible changes in shape could be observed on gigantic liposomes

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containing lipid KAON12 when they were exposed to light of different wavelengths (Bisby et al., 2000b). Briefly, in these liposomes, light triggered the isomerization of azobenzene chromophore moiety. Further, the isomerization of the lipid has initiated changes in the lipid bilayer structure. This shape modification can be easily confirmed by observing under a light microscope as invaginations or protrusions on the surface of gigantic liposomes. The changes are reversible and controlled by light of different wavelengths. In conclusion, the phenomenon could be applicable for triggering of photosensitive liposomes with light (Bisby et al., 1999b; Zhao, 2009).

The success of photo-sensitive liposomal drug delivery entirely depends on several factors such as phototriggerable building blocks of nanocontainer, light source and instrumentation. Apart from photo-sensitive liposomes, another light based therapy i.e. photodynamic therapy is also commonly used to deliver drugs. Photodynamic therapy relies on the activation of photo-sensitizers via light and generation of reactive oxygen species (ROS) that cause the destruction of targeted cells and tissues (Dougherty, 2002; Yano et al., 2011). This therapy is very effective in many tumours and diseases such as tongue cancer in which photofrin acts as photosensitizer (Bicalho et al., 2013), bile duct cancer in which tempoforin acts as photosensitizer (Wagner et al., 2013) and in case of targeting pain in patients, 5-aminolevulinic acid /red light is used (Story et al., 2013). However, photodynamic therapy possesses several limitations such as issues related to the solubility of photosensitizer due to the presence of aromatic and hydrophobic structure, poor bioavailability due to lipophilic nature of photosensitizer etc (Chen et al., 2005; Yavlovich, 2010). Therefore, photosensitive liposomes are beneficial and have many advantages over the photodynamic therapy.

1.3.1. Advantages of photo-sensitive liposomes

As summarised by Shum and co-workers (Shum et al., 2001), photo-sensitive liposomes have the following advantages:

- a) They provide a broad range of adjustable parameters such as wavelength, duration and intensity that can be easily optimized.
- b) Rapid release of the encapsulated contents.
- c) Upon irradiation, photochromic bilayers can lead to membrane reorganization with possible application in imaging, sensing and therapeutics.
- d) Photosensitive liposomes show distinct advantages over photodynamic therapy due to their low toxicity and controllable drug release. This has created new applications in targeted drug delivery such as cancer, eye disease, sun lotion and tissue engineering.

1.3.2. Limitations associated with photosensitive liposomes

Several problems are associated with the efficiency of targeting and with the ultimate fate of light-sensitive liposomes in the body and thus examples of their medical applications are still rare. Clearance of liposomes by macrophages in the liver and spleen reduces circulatory half-life and raises questions of hepatotoxicity and possible immune suppression. Another problem is the penetration of UV light. The UV penetrates into the human body not more than 1-2nm, i.e. superficial layer of the skin. Furthermore, most of the azobenzene based photosensitive liposomes are very toxic to the human body due to their azobenzene derivatives (Leung and Romanowski, 2012).

1.3.3. Synthesis of photosensitive liposomes

Photo-sensitive liposomes have been developed from chemical synthesis by conjugating photo-sensitive groups onto the phospholipid assembly (“designer lipids”). So far, a very few number of designer lipids have been successfully synthesized, each of which employs a definite mechanism for photo activation (Table 1.3) (Yavlovich, 2010). The phosphatidylcholines molecule is composed of three main parts: head group, glycerol backbone and fatty acyl chains (Fig.1.7). With the aim to produce photo-sensitive liposomes, each of these parts has been altered either by inserting additional groups or by modifying the existing chemical bonds such as polymerisable moieties (Chandra et al., 2005; Lamparski et al., 1992a; Lawson et al., 2003; Liu et al., 2005a; Thompson et al., 1996; Yavlovich et al., 2009; Zhang and Smith, 1999).

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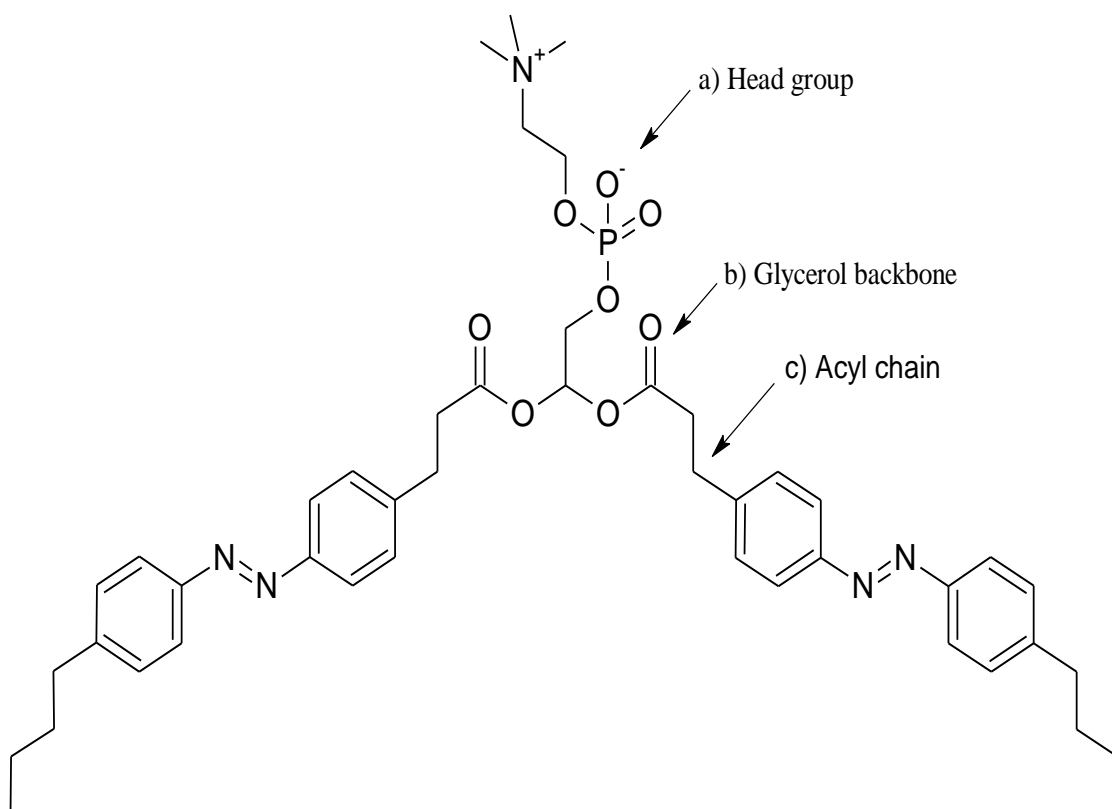


Fig 1.7: Sites for chemical modification in phospholipids.

Table 1.3: Synthesis of Photosensitive liposomes via photoactivation

Lipid modification	Wavelength	Mechanism	Reference
DC _{8,9} PC	254nm	Photopolymerization	(Yavlovich et al., 2009)
Bis-azo PC	UV	Cis-trans photoisomerization	(Liu et al., 2005b)
O-nitrobenzyl conjugated lipid	365nm	Photo-cleavage upon radiation	(Chandra et al., 2005)
NVOC-DOPE	345 nm	Photo-cleavage upon radiation	(Zhang and Smith, 1999)
Plasmalogen	630-820 nm	Photo-oxidation by bilayer sensitizer(Zincphthalocyanine, bacteria chlorophyll a)	(Thompson et al., 1996)

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Lipid modification	Wavelength	Mechanism	Reference
Bis-SorbPC	550 nm	Photo-polymerization induced by Bilayer sensitizer (DiI)	(Lamparski et al., 1992b)
Head-group	UV	Polymerization by radical initiation	(Lawson et al., 2003)

1, 2-bis (10, 12-tricosadiynoyl)-sn-glycero-3-phosphocholine (DC8, 9PC); 6-nitroveratryloxy carbonyl chloride with dioleoylphosphatidylethanolamine (NVOC-DOPE); 1,2-bis- [10-(2',4'- hexadienoyloxy) decanoyl] -sn-glycero-3-phosphatidylcholin (Bis-SorbPC).

Several approaches have been used to design photosensitive liposomes. These involve the photochemical modification of individual phospholipids in the bilayer, i.e. lipid photochemistry, the photoinduced change in the association of polyelectrolytes, the photoiniated polymerization of some or all of the lipids in the liposomes, i.e., photopolymerization and photochemical changes in the structure of individual lipids forming the bilayer. Each technique is briefly reviewed below (Chandra et al., 2006; Yavlovich, 2010) .

1.3.3.1. Lipid photochemistry - photochemical modification of individual lipids in the bilayer

The theory in the design of the photolabile liposomes involves the introduction of a photolabile linker that connects the polar and nonpolar parts of the lipid molecule. Under UV light exposure, the connection between polar and nonpolar parts of the lipid, i.e. photolabile linker, is damaged and the molecule loses its amphiphilic properties, and thus disturbing the bilayer and increasing bilayer permeability (Bennett et al., 1994; O'Brien D. F., 1993). These techniques also include mechanisms such as the trans-to-cis photoisomerization of lipid chromophores, the photoinduced chain cleavage of some or all of the lipids, photomodulated binding of polyelectrolytes, and the photochemical changes in the structure of individual lipids forming the bilayer. The first three are appropriate for phosphatidylcholine (PC) and other phospholipids, while the latter has only been verified with synthetic amphiphiles (Bennett et al., 1994).

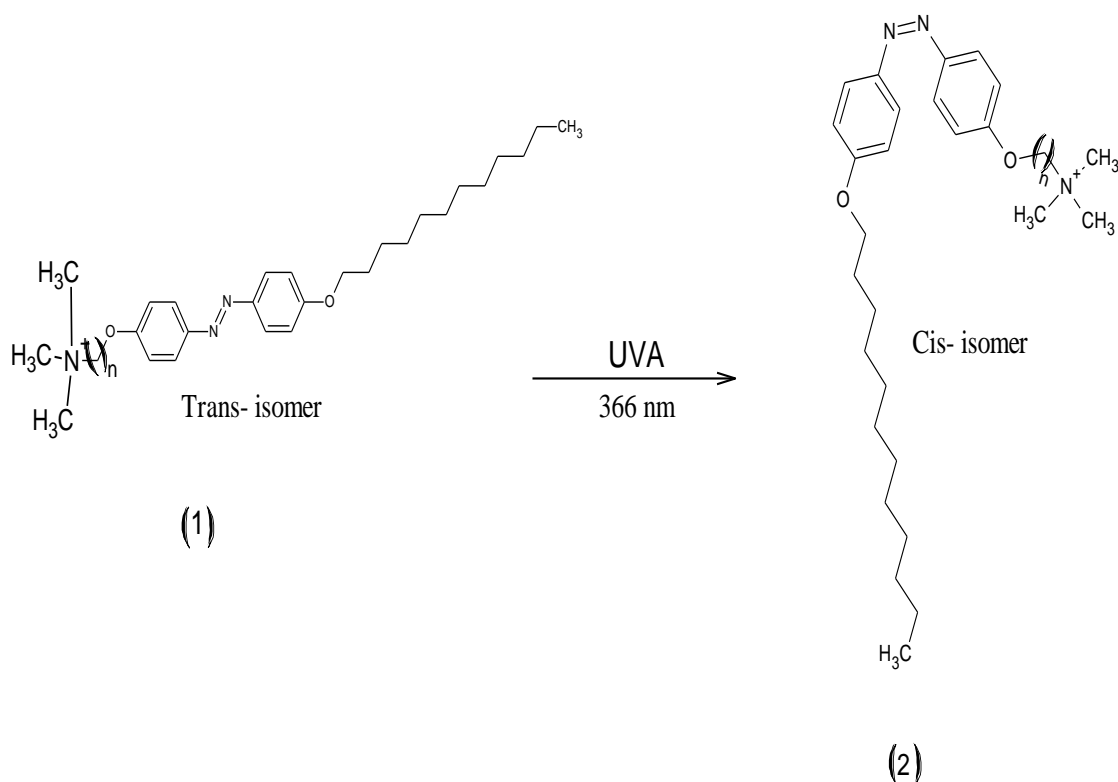
a) Photoisomerization of lipid chromophores-

In order to prepare photosensitive liposomes, both azo and polyenes groups have been introduced into lipid chains. Due to this photosensitive liposomes acts as a molecular switch. A molecular switch is a molecular assembly that disturbs when the system is subjected to an external stimulus, such as light, resulting in conformational and environmental changes of the switch (Merino and Ribagorda, 2012). Therefore, the rationale of a molecular switch is the reversible conversion of chemical species caused through light between two states of a molecule with different absorption

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spectra. For instance, the trans form of azobenzene isomerizes into the cis form of azobenzene by irradiation of light with a wavelength between 330–380 nm. This reaction is reversible and the trans form of azobenzene is easily recovered when the cis isomer is irradiated with light of 400–460 nm, or heated. These photoisomerization processes not only alter the absorption band but also produce differences in different physicochemical properties of molecules, such as molecular geometry, dipole moment, refractive index, electrochemical behaviour (Henzl et al., 2006). In every method light has been successfully utilized to photoisomerize a double bond from trans to cis conformations which perturb the bilayer packing and raise the liposome permeability. The initial experiment in order to affect the membrane permeability using photosensitive lipid was carried out by Kano and colleagues in 1981 (Kano et al., 1981). They effectively incorporated amphiphilic compound 1 (Scheme 1.1) into the bilayer membrane of dipalmitoylphosphatidylcholine liposomes. Cis-isomer of azobenzene 1(2) was experimentally exposed to better maintain the bilayer structure than trans-isomer of azobenzene (1). Under UV exposure the azobenzene molecule undergoes trans-cis isomerization which disturbs the bilayers. For this study, they entrapped bromothymol blue dye inside the bilayer. In its acidic (closed ring) form, this dye is very hydrophobic and remains entrapped within the bilayer. With basification, bromothymol blue gets released into aqueous medium and carries negative charge. After irradiation with 366 nm light, liposomes possessing bromothymol blue (1) were shown to give rapid release of bromothymol blue dye upon basification (see Scheme 1.1).

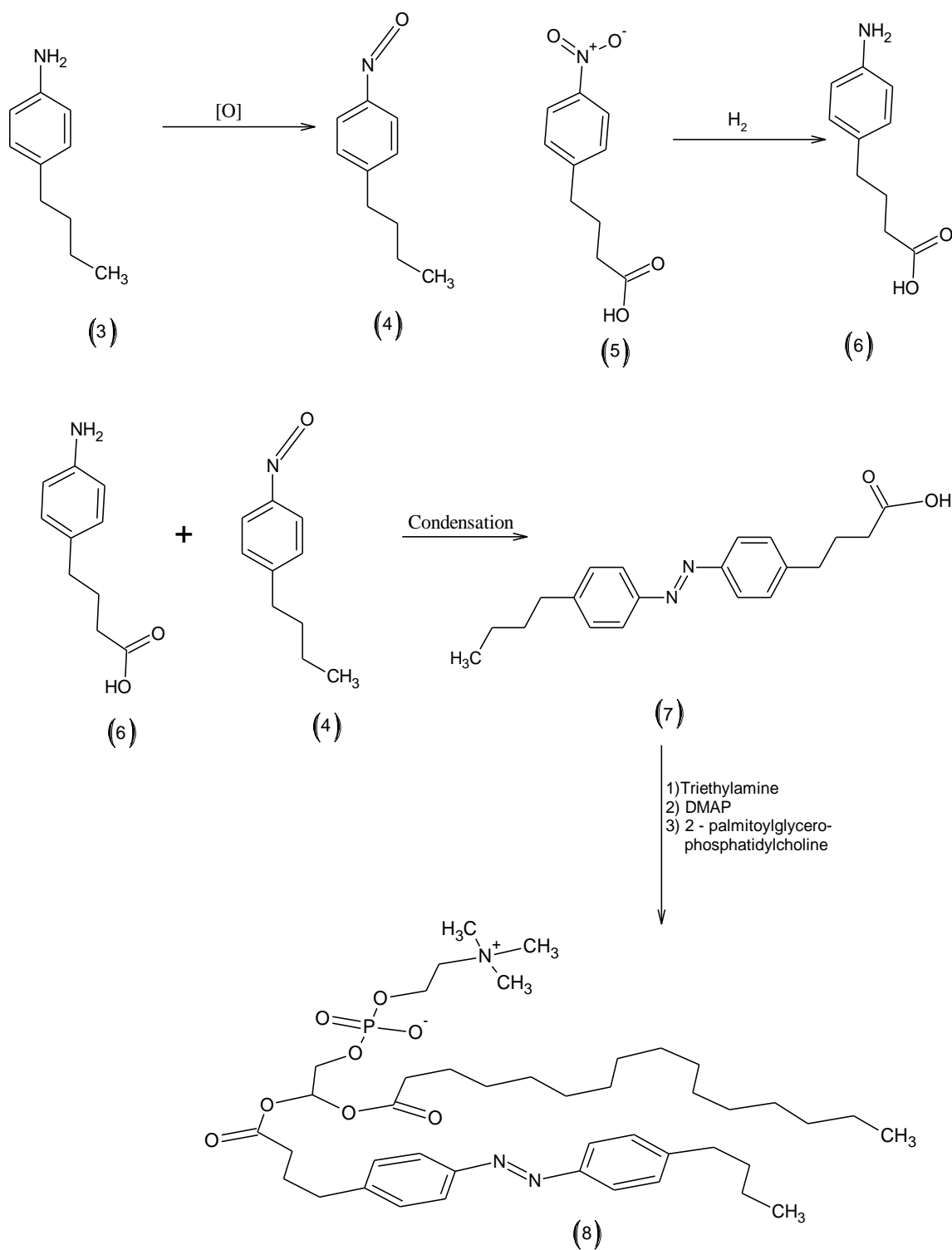
Moreover, osmotic shrinkage experiments also proved that after irradiation, liposomes possessing azobenzene derivative were more permeable to water. In short, this first attempt highlighted the possibility of using synthetic light sensitive amphiphiles to control the membrane permeability with photo irradiation.



Scheme 1.1: Isomerization of first azobenzene derivative [adapted from (Kano et al., 1981)].

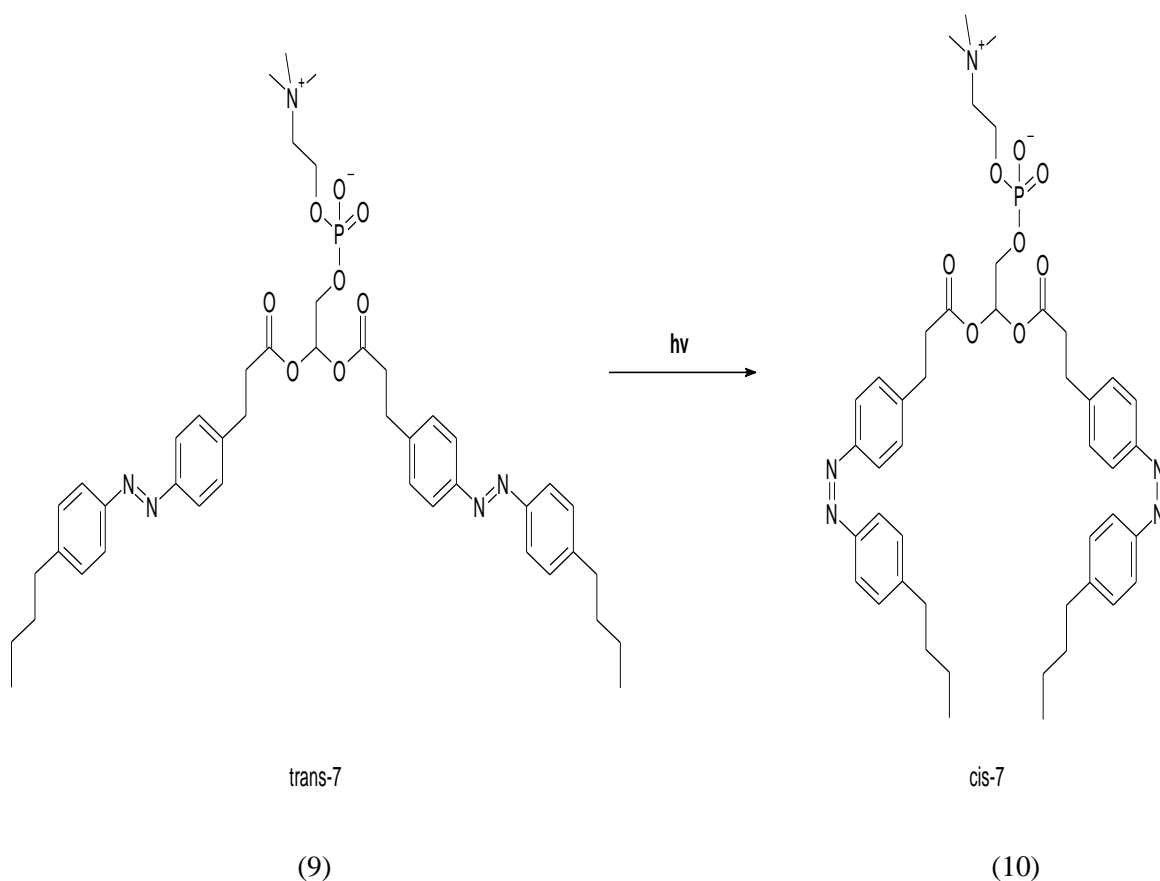
Another interesting work was done by Morgan and his group (Morgan et al., 1985). They used a phospholipid analogue of dipalmitoylphosphatidylcholine. The azobenzene moiety was incorporated into one of the chains of the phospholipid to form Azo-PC lipid (8) (Scheme 1.2). Synthesis of (8) was started with nitroso compound (4) that was obtained by the oxidation of the amine (3), and amino acid (6) that was formed upon reduction of the nitrobenzene (5). Compounds (6) and (4) were combined together to form azo acid (7), which was then converted to Azo-PC (8) by mixed anhydride acylation of 2- palmitoylglycerophosphatidylcholine. Irradiation of compound (8) causes trans-cis isomerization of azobenzene chromophore (Scheme 1.2). Because of linear hydrophobic chains, *trans*-7 shows compatibility with other components of the membrane and is capable of maintaining bilayer stability. Under UV irradiation, *trans*-7 is isomerized to *cis*-7, which perturbs the membrane, thus causing its destabilization and increasing its permeability. Later on, the resulted Azo PC was combined with phosphatidylcholine (6 molar % of Azo-PC) and sonicated to form photosensitive Azo-PC. The results obtained from these liposomes clearly showed that upon irradiation, the permeability of the membrane increased insignificantly. Therefore, light scattering against temperature profiles gave only a slight decrease of phase transition temperature for the irradiated liposomes.

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Scheme 1.2: Synthesis of Azo-PC [adapted from (Morgan et al., 1985)].

Overall, upon UV irradiation, liposomes possessing Azo-PC assembly alter their physical properties such as phase transition temperature and membrane permeability. But, the permeability was insignificantly related to photoisomerization mechanism.



Scheme 1.3: Synthesis of Bis-Azo PC [adapted from (Morgan et al., 1985)].

In a subsequent work carried out by the same authors (Morgan et al., 1985), a new phosphatidylcholine i.e. Bis-Azo PC (9) was prepared (Scheme 1.3). Bis-Azo PC contains an azobenzene moiety in each hydrophobic chain. Upon UV irradiation, this molecule causes trans (9) to cis (10) isomerization of both azobenzene fragments that should lead to more disturbances in the bilayer than observed with Azo-PC. Bis-Azo PC was also synthesized in the same fashion as described for compound (8) (Scheme 1.2). Azobenzene derivative (6) was transformed to mixed anhydride with pivaloyl chloride and then was condensed with glycerophosphatidylcholine to give Bis-Azo PC (Sandhu et al., 1986).

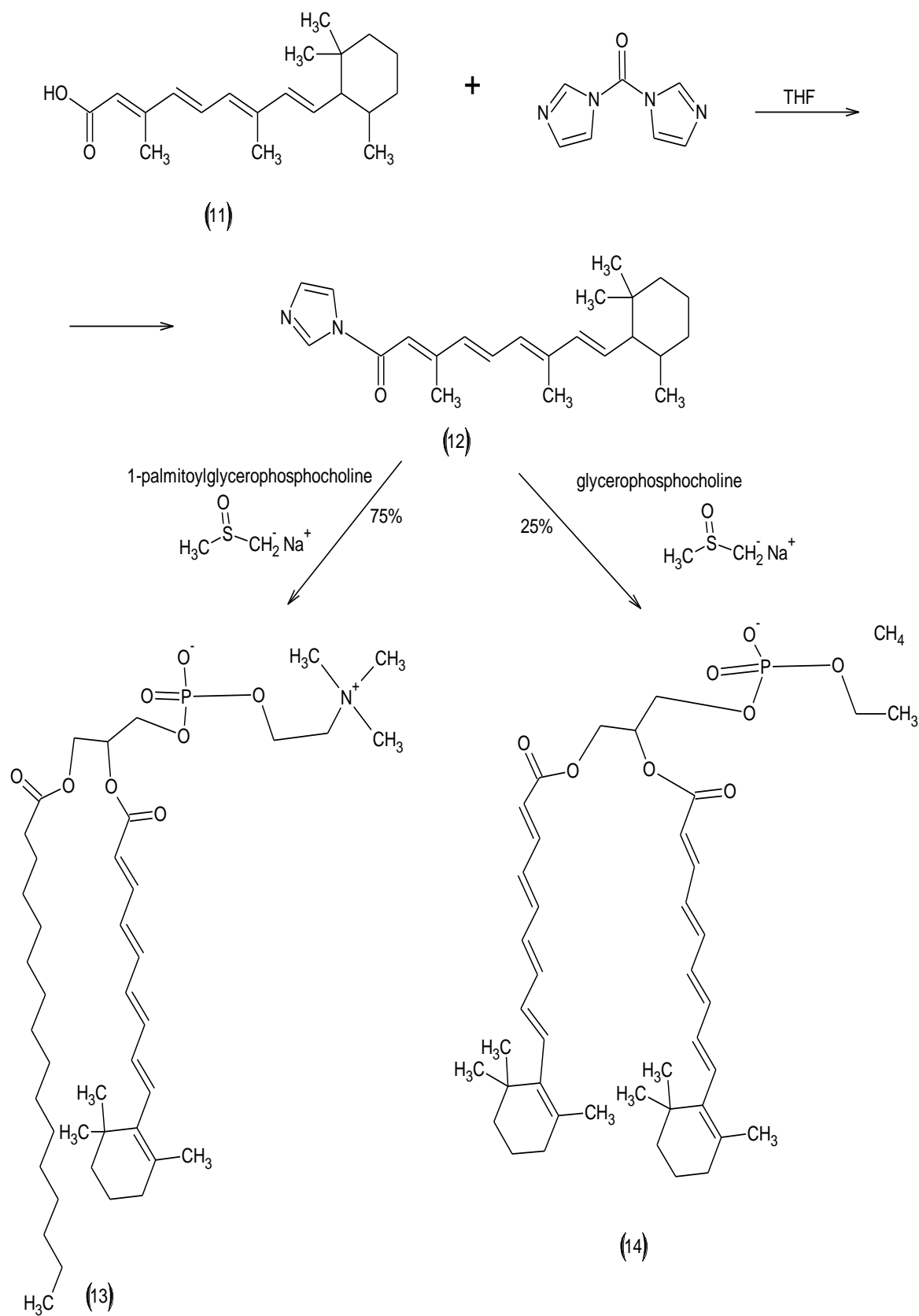
Numerous investigators (Morgan et al., 1985; Morgan et al., 1987b) have described a novel photosensitive lipid system based on a photochromic lipid molecule 'Bis-AzoPC' (1, 2-bis (4-(4-n-butylphenylazo) phenylbutyroyl) - phosphatidylcholine) (Scheme 1.3). This lipid present in a photostationary state is predominantly composed of the trans- isomer which is visible under light illumination, but it is changed to a cis-enriched photostationary state on exposure to near-ultraviolet light. Liposomes of dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine in the rigid 'gel' phase containing up to 5 % of Bis-Azo PC have reserved trapped marker dye for up to several months at room temperature (Morgan et al., 1985). Upon exposure to ultraviolet light at 355 nm,

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conversion of Bis-Azo PC to the cis-photostationary state disrupts the bilayer membrane leading to rapid leakage of contents (Sandhu et al., 1986). Photosensitive lipids might well offer a suitable means to 'cage' water-soluble reagents compatible with the bilayer structure and may supply solutions to the potential problems mentioned above with covalently caged species. A wide number of water-soluble materials including biologically important ions such as calcium and copper and drugs such as methotrexate (Bisby et al., 2000a; Morgan et al., 1987b) can simply be encapsulated under mild conditions without the need for specialised apparatus, and photo-induced release of such solutes from liposomes containing Bis-Azo PC has already been demonstrated (Morgan et al., 1987b). Additionally, liposomes might be appropriate as carriers to introduce reagents into cells by endocytosis, minimising the reagent's exposure to degradative species until release is triggered.

Numerous experiments (Bisby et al., 2000b; Bisby et al., 2000a) have shown that the trapped marker dye, calcein, which is highly fluorescent when dilute, is 'quenched' when trapped at a high concentration within photosensitive liposome. The cited work has shown the effect of phospholipid acyl chain length and temperature on release kinetics of calcein and verified that liposome composition could be tailored to allow for rapid solute release across a range of temperatures. Liposomes of DPPC containing Bis-Azo PC (6 mol %) and cholesterol (up to 15 mol %) showed no significant leakage of calcein for at least 8 weeks. In contrast, liposomes containing higher concentrations of Bis-Azo PC. (e.g., 10 mol %) were stable overnight, but released their contents on long term storage. A further modification of photosensitive liposomes based on photoinduced cis-trans isomerization was projected by Pidgeon and Hunt (Pidgeon and Hunt, 1983). Retinoic acid was effectively incorporated into phospholipid bilayer to serve as hydrophobic chain (12). From this approach, two different types of phospholipids were developed: 1-palmitoyl-2-retinonyl-glycero-3-phosphocholine (13) and 1, 2-diretinonyl-glycero-3-phosphocholine (14) (Scheme 1.4). The methodology used to prepare these two types of phospholipids was developed by Warner and Benson (Warner and Benson, 1977). In this technique, glycerophosphocholine was covalently attached with the imidazolide of retinoic acid in the presence of sodium methylsulfinylmethide.

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Scheme 1.4: Synthesis of 1-palmitoyl-2-retinonyl-glycero-3-phosphocholine and 1, 2-diretinonyl-glycero-3-phosphocholine [taken from (Kulikov, 2006)].

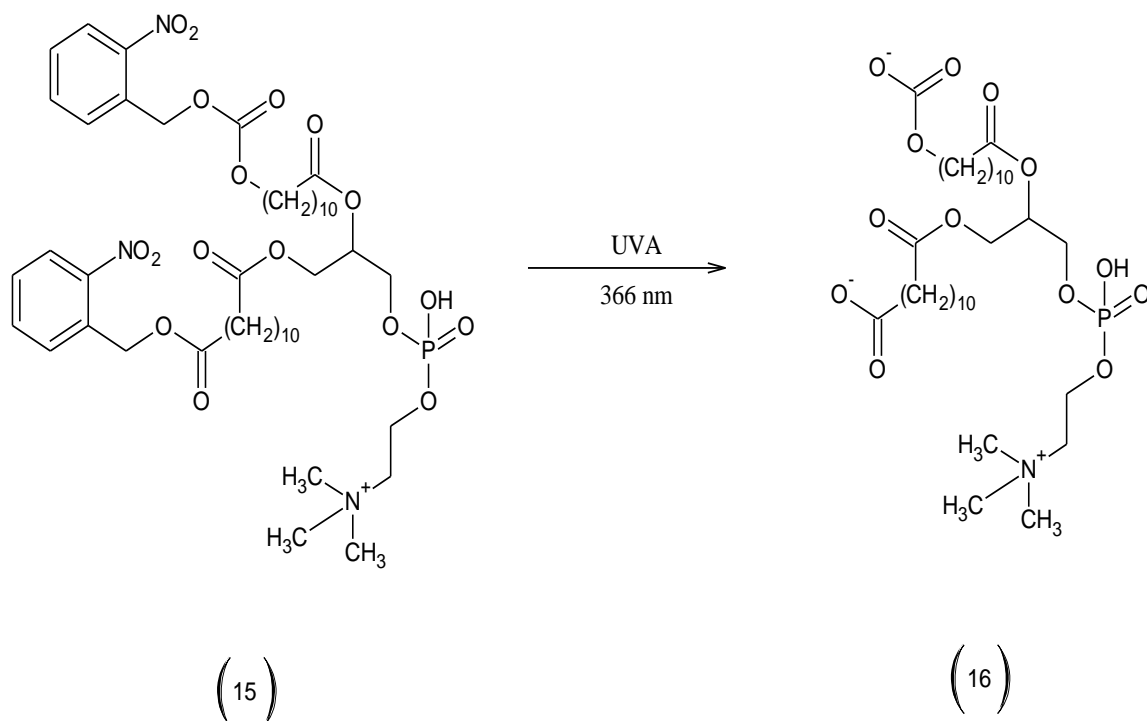
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b) Photoinduced cleavage of lipid chains-

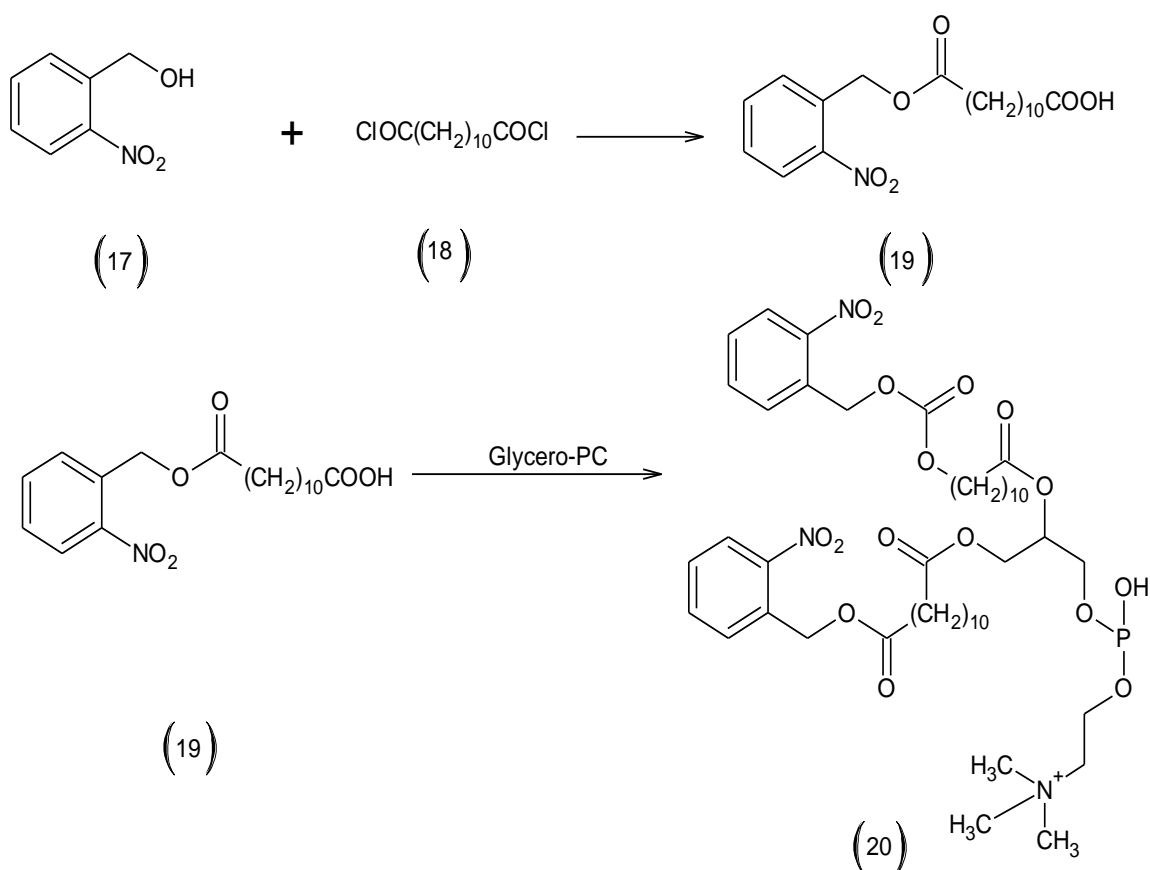
Liposome permeability is altered by the photoinduced cleavage of one or both of the hydrophobic lipid chains which directly reconstructs the bilayer. Two major approaches to the photoinduced cleavage of lipid chains have been reported in previous articles: [1] the direct photoactivated cleavage of designed phospholipids, and [2] the photosensitized cleavage of plasminogen lipids (Bennett et al., 1994).

In 1989, photodisintegratable liposomes data were reported by Kusumi (Kusumi, 1989). Their strategy relied on photoinduced formation of a polar moiety within the hydrophobic chains of the lipid. The investigators prepared phospholipids with 2-nitrobenzyl esters on the terminal carbons of both hydrophobic chains. Under UV irradiation, the 2-nitrobenzyl esters were broken (15) (Scheme 1.5) and two carboxylate ions on the terminal carbons of the hydrocarbon chains were generated (16) which caused the disintegration of the bilayers. As per Scheme 1.6, the phospholipid analogue (20) was developed. Ortho-nitrobenzyl alcohol (17) was mixed with dodecanoyl dichloride (18) to yield ester (19). Further, the ester (19) was coupled with glycerophosphocholine in the presence of dicyclohexylcarbodiimide to give the final product (20).

Scheme 1.5



Scheme 1.5: Disintegration of 2-nitrobenzyl esters via UVA irradiation [adapted from (Kulikov, 2006)].



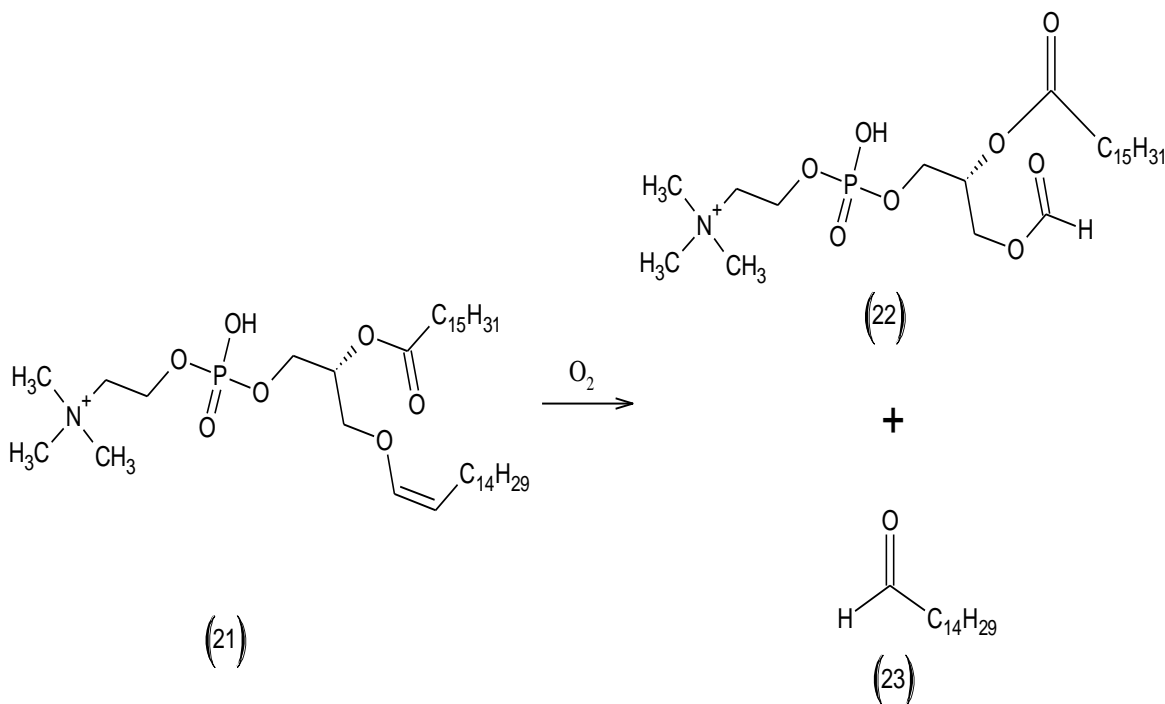
Scheme 1.6: Synthesis of phospholipid analogue of ester [adapted from (Kulikov, 2006)].

Without the addition of natural phospholipids, liposomes were prepared from pure (20). Electron microscopy confirmed the complete disintegration of liposomes upon prolonged irradiation. The results obtained from an experiment on ovalbumin (protein with molecular weight of about 43 kilodalton) entrapped within the liposomes verified that 75 % of protein was released after a brief period of irradiation and barely 5 % of phospholipid underwent the photochemical decomposition. These data suggest that a small fraction of lipid can easily decompose and that caused the disintegration of the liposome (Kulikov, 2006).

During 1992, Anderson and coworkers (Anderson and Thompson, 1992) reported alternative approach to the photoinduced cleavage of lipid chains which was mainly based on the peroxidation of lipids. Moreover, Sensitized lipid photooxidation has been explained for PC liposomes (Grossweiner and Grossweiner, 1982; Kalyanaraman et al., 1987; Viani et al., 1991). Photooxidation of lipid causes cleavage of chains to occur that leads to increase in solute permeability (Kalyanaraman et al., 1987). Anderson and Thompson thoroughly examined the photooxidation of semisynthetic plasmalogen, 1-alkyl-1'-enyl-2-palmitoyl-sn-glycero-3-phosphocholine (21) (Scheme 1.7). This lipid alone or in combination with DPPC

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(9 μ moles:1 μ moles) produced liposomes. A range of photosensitizers were employed in combination with these type of liposomes: the hydrophobic zinc phthalocyanine (ZnPc) with λ_{max} 674 nm, the hydrophilic aluminium chlorophthalocyaninetetrasulfonate entrapped in the liposomes (λ_{max} 672nm) (Kalyanaraman et al., 1987) or membrane bound bacteriochlorophyll a (Viani et al., 1991).

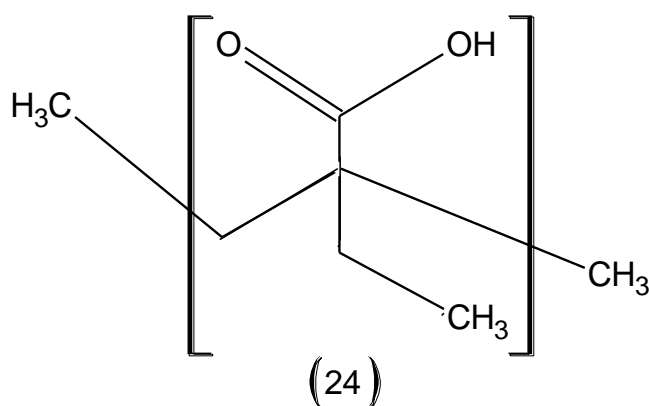


Scheme 1.7: Photooxidation of semisynthetic plasmalogen,1-alkyl-1'-enyl-2-palmitoyl-sn-glycero-3-phosphocholine [adapted from (Anderson and Thompson, 1992)].

c) Photomodulated binding of polyelectrolytes-

The non-covalent attachment of polyelectrolyte chains to lipid bilayers can disturb liposome structures. Tirrell and coworkers have thoroughly studied the reversible association of poly (2-ethylacrylic acid) (PEEA) with PC liposomes (Seki and Tirrell, 1984; Tirrell et al., 1985; Devlin and Tirrell, 1986; Borden et al., 1987; Borden et al., 1988; Maeda et al., 1988; Eum et al., 1989; Laschewsky and Ringsdorf, 1988). The cause was a pH- dependent conformational transition of PEEA in aqueous solutions, from an expanded, highly ionized coil at high pH to condensed globular structures in acidic media (24). pH – dependent conformational transition of PEEA in aqueous solution was exposed by titrimetric, spectroscopic, and scattering techniques (Lohmann and Petrak, 1989; Alvarez-Lorenzo and Concheiro, 2008).

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Poly (2-ethylacrylic acid)

The photomodulated binding of PEEA has been conferred in two ways. In the first approach, PEAA-liposome altered by copolymerization of 2-ethyl acrylic acid with 4 mol % of N-[4-(phenylazo)phenyl]methacrylamide, a light sensitive monomer (Ferritto and Tirrell, 1988). Photoisomerization of the phenylbenzene from the trans to cis changed trans form of the copolymer from a lipid attached polymer to a cis form with a lower surface affinity. The second approach, a water soluble diaryliodonium salt was used as a photoinitiator. Briefly, irradiation of egg pc liposome, PEAA and 3, 3'-Dicarboxydiphenyliodonium hydrogen sulfate at pH 7.7 with 254 nm light results in rapid release of entrapped calcein (You and Tirrell, 1991).

d) Photo-polymerization

Conjugated dienes and their derivatives contain double bonds separated by one single bond. These compounds can be readily polymerised under UV irradiation. In 1992, Lamparsky and O'Brien (Lamparski et al., 1992b) reported an interesting liposome composed of 3:1 molar mixture of dioleoylphosphatidylethanolamine (DOPE) and 1,2-bis [10-(2'-hexadienoyloxy) decarbonyl]-sn-glycero-3-phosphatidylcholine (bis-SorbPC). Upon UV light irradiation, bis-SorbPC undergoes polymerization and gives a cross-linked poly-sorb PC network. This process isolated the liposome bilayer into polymeric and monomeric domains, which resulted in the disruption of the lamellar integrity, liposome fusion and eventually fast release of the encapsulated contents in the liposomes (50% of the contents were released within a few minutes). O'Brien's studies also described that the rate and extent of liposome fusion was dependent on the extent of photo polymerization, the molar lipid ratio of bis-SorbPC, temperature, pH and the presence of Mg^{2+} ions, and that the fusion was initiated by intermediates that associated with the lamellar to Q_2 phase transition of the liposomal membrane (Miller et al., 1996; Mueller et al., 2000; Bondurant et al., 2001; Spratt et al., 2003). Furthermore, studies demonstrated that the fusion rates and extents of DOPE/mono-SorbPC liposomes (Lamparski and O'Brien, 1995). This is due to the fact that photoactivated

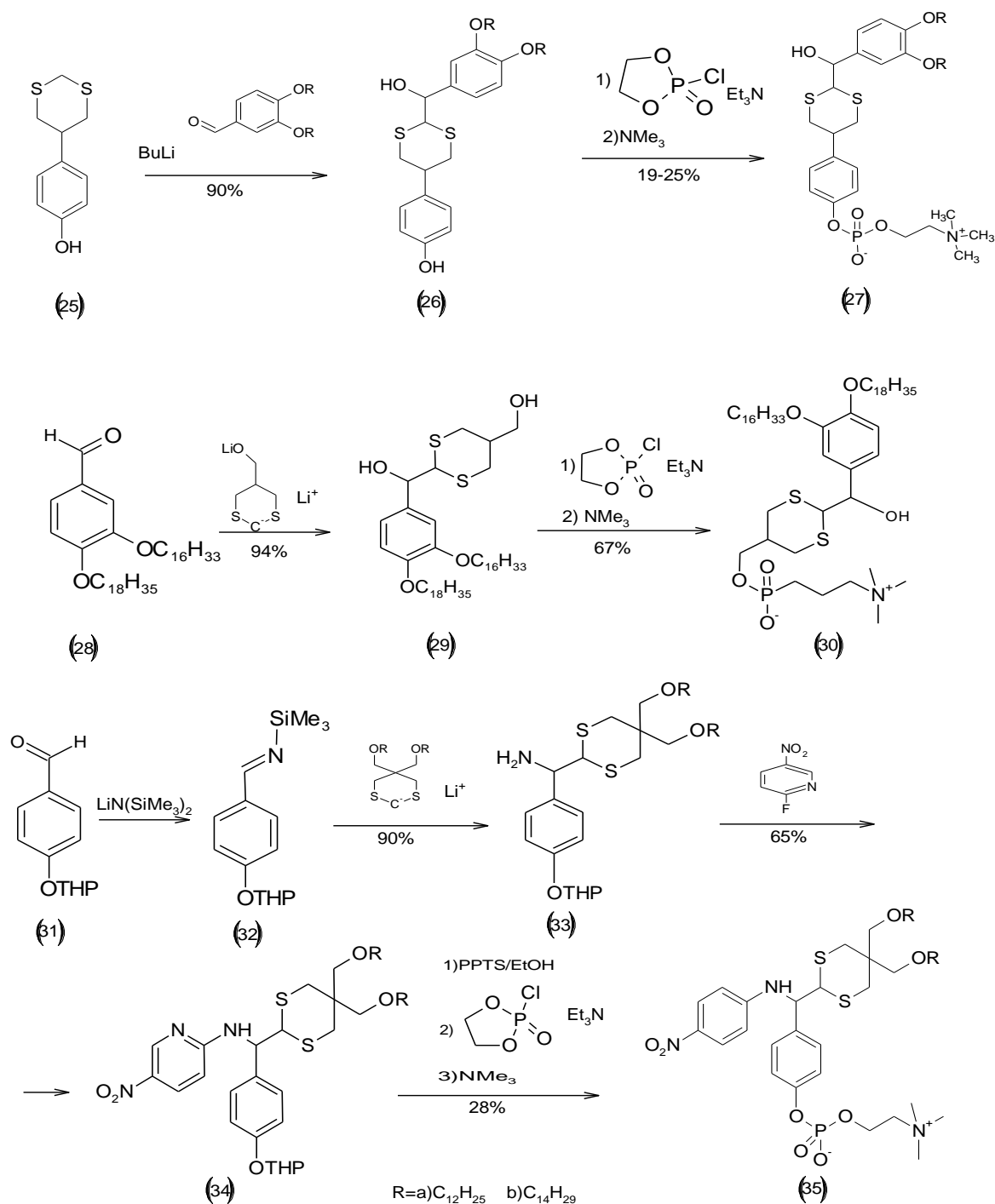
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polymerization of mono-SorbPC produces only short linear oligomers of 3-10 repeat units, and cannot necessarily produce a dramatic phase separation and the subsequent fusion of liposomes. As a result, these studies introduced the possibility of using photo-induced fusion of liposomes to trigger drug release. Taking advantage of the incomparable long circulation life of polyethylene glycol-liposomes (PEG-liposomes also known as stealth liposomes, which have been detected to accumulate preferentially in the interstitium of tumour site because of the increased permeability of the vasculature at those sites), O'Brien et al. further modified DOPE/bis-SorbPC photosensitive liposomes by incorporating PEG-2000 -1,2-distearoyl -sn- glycerol-3- phosphoethanolamine (PEG2000-DSPE) (Spratt et al., 2003). The liposomes were still very sensitive to UV irradiation; 2 minutes UV irradiation promoted a 90% loss of monomer and nearly 60-fold increase in the rate of 1-amino -3,6,8 -naphthalenetrisulfonic acid release from the liposomes (Spratt et al., 2003). Interestingly, the liposomal content release can be triggered even by visible light after incorporation of a green light absorbing dye [distearoylindocarbocyanine, DiIC(18)3] into the bilayer of DOPC/bis-SorbPC/PEG-DOPE liposomes (Mueller et al., 2000; Bondurant et al., 2001; Spratt et al., 2003). These observations demonstrated the possibility of using the available light source suitable for photodynamic therapy to trigger content release from the designed photo-liposomes.

e) Photomodification of the lipid hydrophilic head group-

Incorporation of photolabile linker in the middle of the polar and nonpolar parts of the lipid molecule is an easy technique for the design of photocleavable lipids. These photolabile lipid molecules were designed on the basis of their susceptibility to UV light resulting in the breakdown products that will destabilize liposome membrane. Initially, this type of photosensitive liposomes was designed by the Kutateladze group (Wan et al., 2002). Later on, synthesis of a photocleavable lipid based on a dithianephotosensitive linker was carried out by Li and co-workers (Li et al., 2003). These authors also reported the synthesis of a number of photocleavable amphiphiles based on a dithiane moiety. Scheme 1.8 represents the synthetic cycle of some of these phospholipid analogues. Basically, synthesis of these amphiphiles initiated by a coupling reaction between an appropriate aldehyde and the lithium salt of the dithio compound. Then, the free phenolic hydroxyl is esterified with phosphocholine upon treatment with cyclic chlorophospholane followed by trimethylamine. All those photocleavable amphiphiles were revealed as effective materials for the development of stable liposomes and no leakage was detected in the dark due to the absence of light.

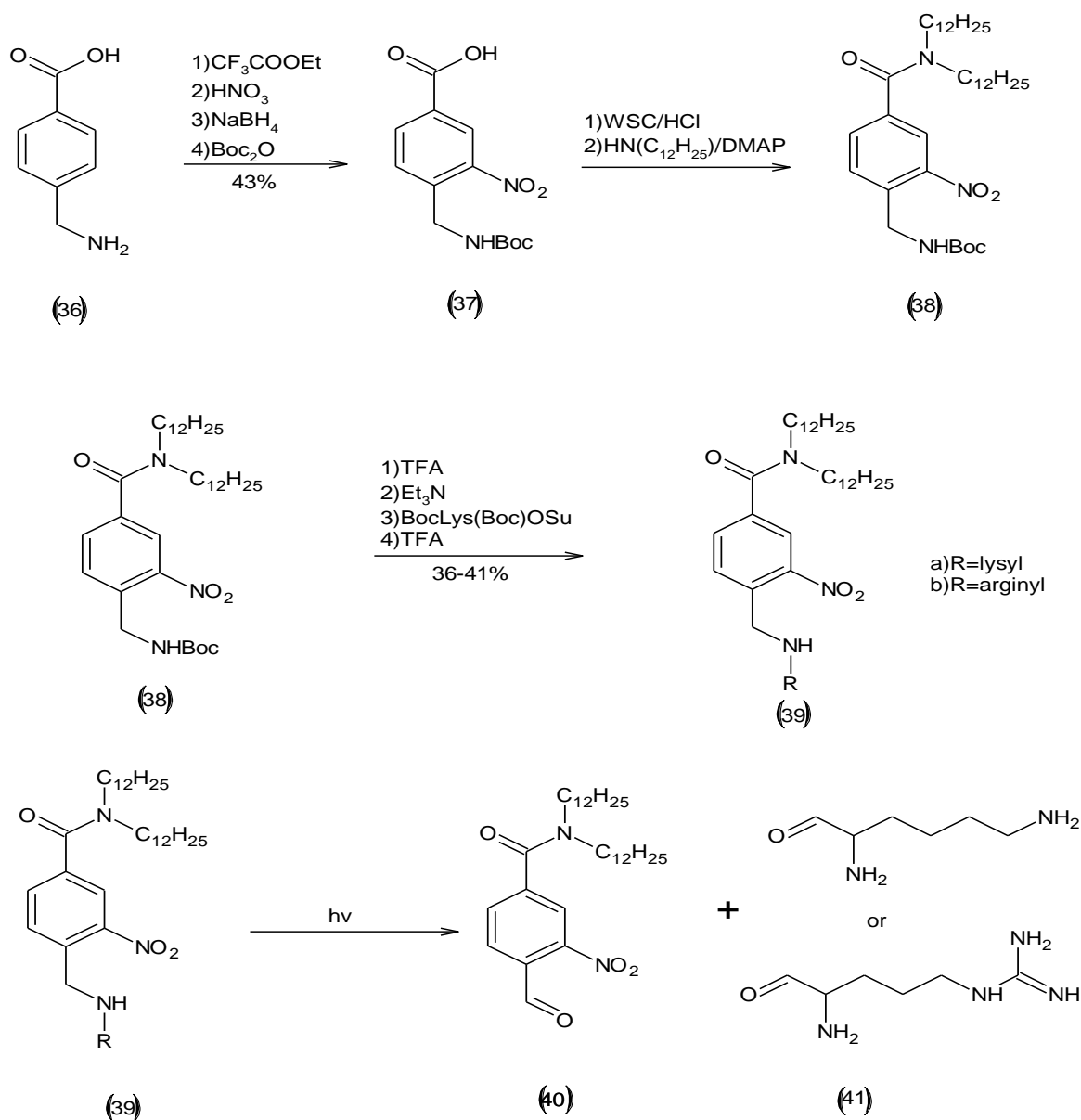
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Scheme 1.8: Synthetic cycle of a number of photocleavable amphiphiles based on a dithiane moiety [taken from (Kulikov, 2006)].

In 2002, Nagasaki and his group (Nagasaki et al., 2003) had attempted to use photocleavable lipid analogs for the preparation of liposomes that could be used as gene delivery systems. With the aim to synthesize photocleavable lipid, they incorporated photolabile ortho-nitrobenzyl moiety between the polar and nonpolar parts of the lipid molecule (Scheme 1.9).

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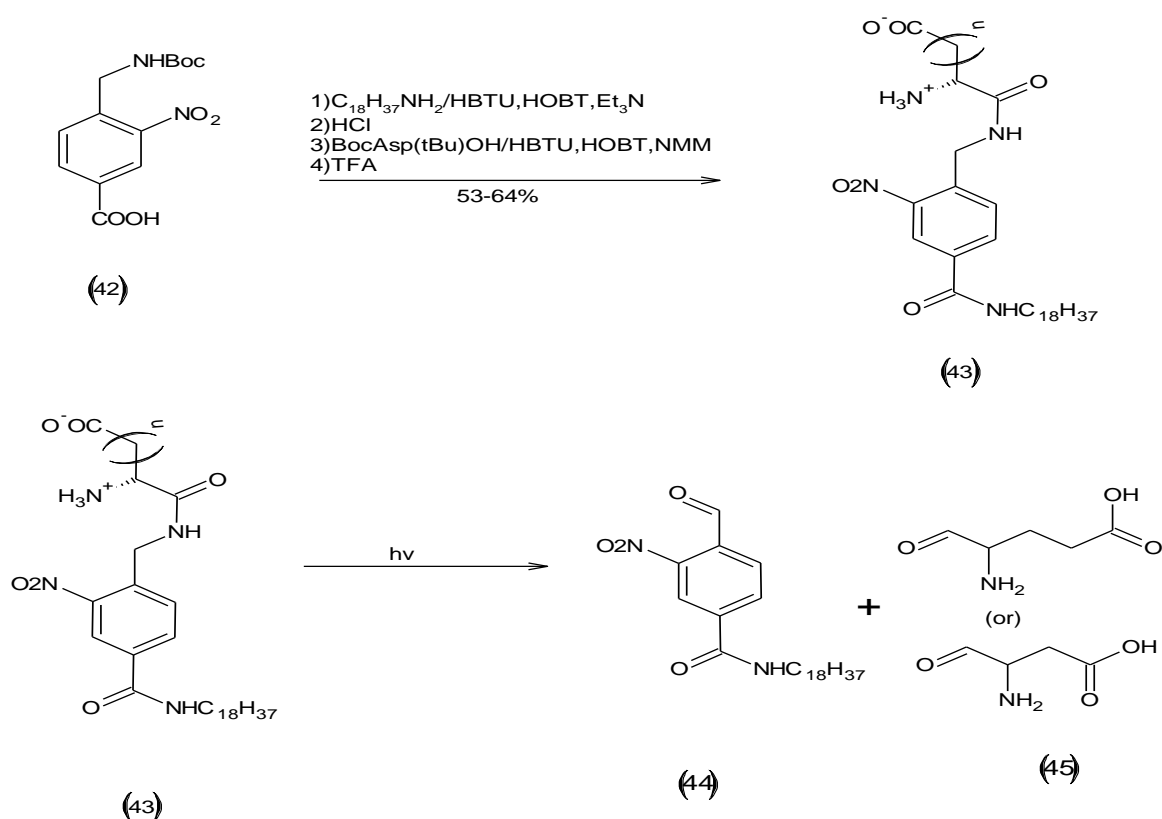
Scheme 1.9: Design of photocleavable lipid by incorporating photolabile ortho-nitrobenzyl moiety between the polar and nonpolar parts of the lipid molecule [taken from (Kulikov, 2006)].

Hydrophobic chains of this lipid analogue are depicted by two dodecyl hydrocarbon chains, while the polar head group of the lipid is composed of basic amino acids lysine or arginine, in the place of phosphocholine. Synthesis of lipid analogue (39) begins with an initiator i.e. amino acid (36) (Scheme 1.9). The amino acid (36) was nitrated and the amino functionality was protected with tert-butyloxycarbonyl derivative to give carboxylic acid (37). Then, coupling reaction occurred between the carboxylic acid (37) and didodecyl amine. On account of this, the tert-butyloxycarbonyl protection was cleaved. The product thus obtained was mixed with lysine (a) or arginine (b). Final deprotection then resulted in the desired product (39). Under UV irradiation of amphiphiles (39),

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the whole molecule lost its amphiphilic characteristics and the amino acids were released as amines.

With UV irradiation, the liposomes prepared with compounds (39) showed higher transfection activity than that of liposomes prepared with lipofectin (commercially available mixture of lipids for liposome preparation). A similar strategy to design photocleavable lipid was reported by Chandra (Chandra et al., 2005; Chandra et al., 2006). A photocleavable lipid with only one hydrophobic “tail” was the basis of their study and acidic amino acids such as asparagine and glutamine represented the polar part of the lipid. The complete cycle of synthesis of this lipid analogue is illustrated in Scheme 1.10. The synthesis cycle of lipids (43) is very similar to synthetic cycle of compounds (39). But, the yield of obtained lipid (43) was 53 %.



Scheme 1.10: Synthesis of photocleavable lipid with only one hydrophobic tail [taken from (Kulikov, 2006)].

Following the synthesis of photocleavable lipids (43), photosensitive liposomes were prepared with 95% yield (by weight) 1, 2-distearoyl-glycero-3-phosphocholine and 5% photocleavable lipids (43). Further study with photosensitive liposomes entrapped with fluorescent marker demonstrated efficient release of liposomal contents upon UV irradiation and no leakage was detected in the dark due to absence of light.

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All these are methods that are commonly used to synthesize photosensitive lipids. As mentioned earlier, the initial objective of this research has been to synthesize photosensitive liposomes. For that reason, in this investigation, lipid photo chemistry method has been used. (as explained in the lipid photochemistry method section 1.3.3.1a) and formulated photosensitive lipid i.e Bis-Azo PC that has been further incorporated into the liposomes (DPPC:Chol) which are described in detail in Chapter 3 and Chapter 4.

1.3.4. Application of photo-triggerable formulations in drug delivery

The first attempt to release doxorubicin from PEGylated liposomes with the help of laser irradiation was done by Yavlovich and co-workers (Yavlovich et al., 2011). In that study, doxorubicin entrapped PEGylated liposomes were formulated by conjugating doxorubicin entrapped DPPC liposomes with 10% of the photopolymerizable diacetylene phospholipid (DC_{8,9}PC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] and then this was used *in vitro* for cell toxicity studies. A photo-triggerable release of doxorubicin was achieved by laser at 514 nm wavelength. Compared to unreacted cell in culture, laser treated culture of doxorubicin loaded liposomes and cells had showed 2-3 fold improvement in cells toxicity (Yavlovich et al., 2011). This finding provides the basic platform for photo-triggerable formulations that are clinically suitable for skin cancer drug delivery (Perche and Torchilin, 2013). In terms of photodynamic therapy, targeting efficiency of folate-targeted liposomes have been achieved by incorporating photosensitizer i.e. zinc tetraphenylporphyrin (ZnTPP) into folate-targeted liposomes (Garcia-Diaz et al., 2011). Data obtained from the study clearly showed that this modification has not only increased the targeting efficiency, but also improved the photocytotoxicity of folate-targeted liposomes. However, phototoxicity of folate – targeted liposomes was completely inhibited with an excess of folic acid present in the cell culture media. Moreover, O₂ kinetics analysis in living cells clearly indicated the presence of ZnTPP in lysosome. Light sensitive liposomes have also been used to entrap the third generation photosensitizer i.e. m-THPC [5, 10, 15, 20-tetra-(m-hydroxyphenyl)chlorin] which is formulated by using dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol (DPPC/DPPG) liposomes encapsulating m-THPC (Lassalle et al., 2009). This light sensitive liposomal material is termed as Foslip and has showed improvement in photodynamic activity (Lasalle et al 2009). Moreover PEGylated liposomes have been used as nanocontainer for the same photosensitizer i.e. m-THPC [5, 10, 15, 20-tetra-(m-hydroxyphenyl)chlorin] (Bovis et al., 2012). Results obtained from the study clearly indicated that higher tumour photocytotoxicity was achieved via m-THPC incorporated PEGylated liposomes in comparison to standard m-THPC formulation (Foscan®). In theory, normal tissue damage has reduced and tumour uptake has increased with higher dose of m-THPC incorporated PEGylated liposomes (Bovis et al., 2012; Perche and Torchilin, 2013).

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To date, a large number of light sensitive formulations have been developed with the help of liposome based strategy and are being used for photodynamic therapy and imaging. Table 1.4 illustrates some examples of light sensitive formulations (Loomis et al., 2011; Zharov et al., 2005) .

Table 1.4: Examples of photosensitive formulations

Photo-sensitive formulations	Reference	Clinical status	Photo-sensitive moiety	Purpose
C- dots (carbon dots)	(Huang et al., 2012; Huang et al., 2013)	<i>in vitro/in vivo</i> studies	metal ions	theranostics
functionalized gold nanostars	(Wang et al., 2013)	<i>in vitro/in vivo</i> studies	Photosensitizer (chlorine 6)	PTT/PDT
tuneable plasmonic nanobubbles	(Anderson et al., 2010)	<i>in vitro/in vivo</i> studies	Gold nanoparticle	Drug delivery
light responsive Polymer nanoreactors	(Baumann et al., 2013; Tanner et al., 2011)	<i>in vitro</i> studies	photosensitizer-protein conjugate	Production of Reactive Oxygen Species (ROS)
Polymers	(Fomina et al., 2010; Fomina et al., 2011)	<i>in vitro/in vivo</i> studies	Photopris phthalocyanines	neovascularization
Dendrimers	(Nishiyama et al., 2007; Nishiyama et al., 2009)	<i>in vitro</i> studies	Porphyrin-phthalocyanine	Drug delivery
Liposomes	http://www.visudyn.com	in clinics (visudyne)	verteporfin	Delivery of photosensitizer

Recently, a novel photo-triggered release system based on metal ion has been successfully developed, which involves the use of carbon dots (C-dots) and photosensitizer-functionalized gold nanostars in order to achieve the photo-triggered theranostics (Bovis et al., 2012; Huang et al., 2012; Huang et al., 2013; Perche and Torchilin, 2013; Wang et al., 2013). Another marvellous achievement in case of light triggered formulation has been obtained by Lin and colleagues using Ce6-loaded gold vesicles demonstrating both photothermal and photodynamic effects (Lin et al., 2013). Furthermore, light triggered formulation using tuneable plasmonic nanobubbles has been used to disintegrate the lipid vesicles and to achieve the controlled release of drugs (Anderson et al., 2010). This formulation possesses gold nanoparticles that produce the vapour bubble through short laser and the vapour bubble disrupts the liposomes. This formulation also shows the beneficial *in vivo* application in case of zebrafish. However *in vivo* studies on mammals are in yet to be carried out.

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Photosensitive polymer has also been developed using quinone-methide (2, 6-bis (hydroxyethyl)-p-cresol), self-immolative dendrimer as photosensitive components and which show triggered release of drugs through near infra-red (NIR) light (Fomina et al., 2010; Fomina et al., 2011). Another noticeable nanosystem for drug delivery is produced by loading photosensitizer-protein conjugate along with polymers known as “light responsive polymer nanoreactor” that is easily triggered through light and produce highly reactive oxygen species (Baumann et al., 2013; Tanner et al., 2011). Nowadays, photo-sensitive dendrimers and polymeric micelles are on demand for drug delivery and these are produced by using porphyrinphthalocyanine as photo-sensitive component (Nishiyama et al., 2007; Nishiyama et al., 2009). Even though all these light sensitive formulations are widely accepted as potential tool for photodynamic therapy in the case of cancer drug delivery, there is only one successful and clinically approved drug for photodynamic therapy, which is Visudyne®. Visudyne® is light sensitive liposome formulation which possesses the photosensitizer verteporfin (benzoporphyrin derivative monoacid ring A, BPD-MA) and is highly beneficial in treating age-related macular degeneration disease (AMD). Visudyne® is mainly comprised of BPD-MA: Egg phosphatidylcholine- glycerol:dimyristoyl PC in the ratio of 1:05:3:5. This light sensitive liposome was invented by Novartis and clinically approved by FDA in 2000.

Apart from photodynamic therapy formulations, light sensitive liposomes are also used for other therapies. One of the well-known example is the use of photosensitive liposomes for tissue engineering (Smith et al., 2007). In this study, photosensitive liposomes were developed by using the photosensitive lipid (1, 2-bis (4-(4-n-butylphenylazo) phenylbutyroyl) phosphatidylcholine) i.e. 'Bis-Azo PC' which is triggered by long wavelength ultra violet light at 385 nm. With UV irradiation, release of calcium chloride from liposomes occurs that causes cross linking of alginate solution and immobilisation of bone-derived cells that support 3D tissue construction. Therefore, this therapy plays an important role in the development of tissue engineering scaffold (Smith et al., 2007).

In conclusion, photosensitive liposomes have many possible uses in the biomedical field and have also been investigated as potential nanocontainer (Yavlovich, 2010). However, much research in the recent years has focused on cancer and other types of drug delivery. Photosensitive liposomes are also used for cosmetic purposes. But very few articles have reported the use of photosensitive liposomes for treating skin diseases. Most of the time photosensitive liposomes are used for cosmetic purposes. Morgan and co-workers (Morgan et al., 1991) has addressed the release of methotrexate from photosensitive liposomes and highlighted the application of these liposomes in case of psoriasis treatment. Therefore, another objective of this project is to trigger the release of psoralene derivatives from photosensitive liposomes in order to treat psoriasis conditions. From this, the question “what is psoriasis” arises?

1.4. Psoriasis

Psoriasis is an inflammatory skin disease in which the human immune system mistakes a normal skin cell for a pathogen, and sends out defective signals that cause overproduction of new skin cells. It is also a chronic disease that is signalized by hyperkeratosis and epidermal hyperplasia of varying degrees (Gennaro, 2006). The epidermal patches are red and scaly. These scaly patches are found on the external surfaces of skin of the knees, elbows, back region and scalp. Furthermore, nails become pitted and ridged. Psoriasis is categorized in the minor disease category. However, generalised form and systemic manifestation can be determined (Male, 2013). Fig 1.8 illustrates the difference between psoriatic and healthy skin



Fig 1.8: Difference between psoriatic and healthy skin

[taken from <http://www.fitnessremediesdiets.com>]

1.4.1. Epidemiology

Approximately 1-2% of individuals in the UK possess some forms of psoriasis. In USA, this figure is about 1-3% (Gennaro, 2006). Northern European countries show a higher number of psoriasis patients. It depends on races. Both males and females are affected in equal rates. That is why psoriasis does not come under the social class linked disease (Farber and Nall, 1974). Psoriasis can occur at any age. Most dominant stages are in early and middle adulthood.

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1.4.2. Etiology

The exact prevalence of psoriasis disease is not known. However, most of the research work has proved that psoriasis is linked to hereditary genes (RDR, 1998). It is generally believed that heredity plays a very important role in this disease and these genes are induced and exaggerated by various environmental factors such as decreased humidity, an injury to skin through cutting, sunburns or insect bites (this whole mechanism is termed as Koebner response), smoking, stress and throat infection (Henseler and Christophers, 1995). Furthermore, psoriatic conditions linked with nutrition can range from nutritional deficiencies, excess nutrients or metabolic disorders (Basavaraj et al., 2010).

1.4.3. Pathogenesis

Psoriasis is mainly characterized by three features- (Griffiths et al., 2000)

- 1) Hyperkeratosis and epidermal hyperplasia of varying degrees.
- 2) Epidermal vascular hyper-proliferation and differentiation.
- 3) T cell mediated inflammatory infiltrate of the intra-dermal and epidermal layers.

It is widely accepted that psoriasis is an autoimmune disease that comes under the category of T cell-mediated diseases. The autoantigen which causes psoriasis condition is a mystery. Even though an epidermal protein such as keratin 17 is mainly considered as speculative superantigen (Ellis et al., 1991), T cells, which are considered as the immunity wall of human system, are responsible for psoriasis. In theory, due to superantigens induction, T cells start to attack the healthy skin. As a result human body produces large number of new skin cells and T-cells. This cycle of skin and T-cell production increases day by day. All these cells survive for up to five to six days rather than 28 days and then dead skin cells thicken the dermal surface of skin in the form of scaly patches. Furthermore, this T-cell mediated reaction increases the release of T-helper 1('Th1') cytokines such as IL-2 (Interlukin-2), IL-12, interferon- γ and T-helper 2('Th2') cytokines such as IL-4, IL-5, IL-10 (Fry, 1988; Voorhees, 1996). Because of this hormonal imbalance features, psoriasis is always associated with other T-helper 2 ('Th2') related diseases such as asthma, atopic dermatitis and urticaria. This whole mechanism is scientifically proven through an experiment in which psoriasis is effectively treated by T cell-targeted therapy (Elias, 1983; Gottlieb, 1995). In this experiment, drugs such as cyclosporine and an interleukin 2 (IL-2) fusion toxins were used to treat psoriasis.

In summary, the pathogenesis of psoriasis may give ideas to develop targeted therapies for psoriasis treatment.

1.4.4. Treatment of psoriasis

Treatment of psoriasis involves two main therapies: first line therapy and second line therapy (Griffiths et al., 2000). First line therapy involves the use of active ingredients such as emollients, coal tar, Dithranol and various forms of topical steroids. Route of administration for this category is topical and all these active ingredients are delivered in the form of ointments and creams (RDR, 1998). In the UK, most of the psoriasis cases are treated by first line therapy only the most common side effects of all these topical preparations are irritation of uninvolved skin, inflammation of hair follicles, telangiectasia, stretch marks and Cushing's syndrome (Hypocorticism in case of corticosteroids) (Clark et al., 1998). But the severe and extensive forms of psoriasis (pustularpsoriasis) are insensitive and are not controlled by topical therapy. Therefore, a physician would recommend second line therapy which involves the use of the phototherapy or light therapy along with drugs. Second line therapy takes place in clinics under the supervision of doctors or clinicians (Gawkrodger, 1997). Phototherapy involves the irradiation of human skin with UV light on a regular basis under medical supervision. Two types of UV light may be given, using special machines: ultraviolet A (UVA) which possesses wavelength between 400–315 nm and ultraviolet B (UVB) which retains wavelength between 315–280 nm (Krutmann, 1998). These are different parts of normal sunlight. Human skin is sensitive to UVB and psoriasis can be easily treated with UVB exposure (Goeckerman, 1931). In contrast, UVA directly penetrates to the body and requires sensitizing medication such as psoralen to treat psoriasis. This process is known as PUVA (combination of psoralen drugs and long wavelength ultraviolet A) (Roelandts, 1991). There are several routes of administration for taking psoralen drugs in case of PUVA therapy. First is oral route in which the patient can take psoralen drug in capsule two hours before the treatment with UVA light. Alternatively psoralen can be added to bath water (bath PUVA). Lastly is the topical application of psoralen gel or cream to small areas of psoriasis, about half an hour before UVA light treatment (Gawkrodger, 1997).

There are several drawbacks of phototherapy for the treatment of psoriasis as summarised below:

- 1) Both UVB and PUVA therapies increase the risk of skin cancer.
- 2) In case of PUVA therapy, UV-sensitizing effects of psoralen persist for several hours. So after PUVA treatment, patients must protect their skin and eyes from sun exposure for about 24 hours.
- 3) Both UVB and PUVA therapies cause erythema and nausea.

In order to provide effective treatment and reduce the limitations of first line and second line therapies, a few researchers have used photosensitive liposomes as nanocontainers to treat psoriasis

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(Morgan et al., 1991). But they have used MLV. Apart from this, here in this project, IFV for the controlled release of psoralen derivative i.e. 4'-(6-Hydroxyhexyloxy) methyl-4, 5', 8-trimethylpsoralen (HMT) has been used in order to treat psoriasis. This IFV offers many advantages.

- 1) Clinical trial study shows that higher doses of psoralen work better to clear psoriasis than lower doses (Griffiths et al., 2000). However high dose means more side effects. Through photosensitive IFV it is possible to achieve a “burst release effect” therefore, this strategy can provide higher amount of psoralen to the affected area within a short period of time.
- 2) Photosensitive IFV suspension can be applied topically and easily absorbed through skin and will remain in the human epidermis. By exposing to long -wavelength UVA light the entrapped psoralen is released from photosensitive IFV. After finishing this treatment, there is no need for the patients to protect their skin and eyes from sun exposure for 24 hours (h) because in this treatment psoralen entrapped-IFV can be applied topically. According to the Neild and Scott (Neild and Scott, 1982), psoralen via topical route not only rapidly penetrate the skin but also detected in the urine after 4 hours whereas psoralen taken via oral route has a serum half-life of approximately 1 hour, however, the skin remains sensitive to light for 23 hours.
- 3) It is a novel method and utilisation of UVA radiation is very less when compared to PUVA treatment.
- 4) Erythema and nausea side effect observed in PUVA therapy can be minimised by photosensitive IFV, as IFV acts as barrier for the release of psoralen and thus the human skin is not directly exposed to psoralen compound.

In this way, Photosensitive IFV along with PUVA therapy may be beneficial and in future it should become an important tool in treatment of psoriasis. As discussed earlier psoriasis is an inflammatory skin disease that is known to arise from the interplay of both genetic and environmental factors such as skin injury, stress and smoking which trigger excess skin inflammation resulting in psoriatic plaque (epidermal hyperplasia). In order to better appreciate predisposition to psoriasis, its etiologic link to skin, a better understanding of structure and behaviour of the human skin is necessary. In addition, an understanding of the skin structure and skin functions are sought in order to develop novel drug delivery tools improving on drug delivery efficiency and therapy efficacy.

1.5. Human skin

The human skin covers the outer part of human body and is considered the largest organ of the human integumentary system. It is anatomically useful in containment of fluids and tissues, acts as an indispensable barrier to microorganisms and noxious chemicals, allergens and UV radiation and regulates heat and water loss (Proksch et al., 2008). Human homeostasis mechanism is entirely controlled by the skin. The skin of a 70 kg adult weighs about 3.6 kg and covers a total area of about 22 square feet (Williams, 2003).

1.5.1. Skin Anatomy

Anatomically, the human skin can be categorised into three main layers: - the epidermis, the dermis and the subcutaneous fatty tissue along with skin appendages, such as hair follicles, sebaceous and sweat glands (Bouwstra et al., 1991; Bouwstra et al., 2003; Tortora, 2008). Fig.1.9 illustrates the representation of human skin.



Fig 1.9: A representation of human skin [taken from <http://healthfavo.com/labeled-skin-diagrams.html>].

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Epidermis is the outermost layer of skin which functions as a waterproof barrier and constructs our skin tone (Williams, 2003). Structurally, epidermis is made up of keratinocytes (~91%), merkel cells (5-9%), melanocytes and Langerhans (Kanitakis, 2002). This layer neither holds any blood vessels nor connects to blood capillaries. Therefore, for nourishment, epidermal cells entirely depend on the dermis layer (Stucker et al., 2002). The epidermis is a vascular complex that consists of five sublayers which are: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum germinativum (Tortora, 2008).

Stratum corneum is the superficial layer of epidermis and is termed as a 'horny layer' which gives the skin its waterproof barrier properties (Bouwstra et al., 1991). Stratum corneum is made up of dead keratinocyte cells (Kanitakis, 2002). Keratinocytes are responsible for synthesis of keratin and have tendency to divide approximately every 28 days. Thus, keratinocytes play a major role in skin repair (Wilkes et al., 1973). They also diminish the transepidermal water loss and provide protection against the pathogens and chemicals. Several researchers points out that stratum corneum is a two compartment model, brick and mortar, in which keratinocytes are the bricks and the lipid lamellae represent the mortar (Forslind et al., 1997; Elias, 1983). In theory, this superficial layer serves as the main barrier to the body and thus is a major hurdle in skin drug delivery (Wertz, 2000; Laugel et al., 2005). Stratum lucidum is situated in between the stratum granulosum and stratum corneum, and entirely made up of flattened keratinocytes. It has a translucent appearance which is easily seen through microscope (Flynn and Woodhouse, 2009). Stratum lucidum is also present in thick areas of skin such as palms of the hands and the soles of the feet (Mata and Dissanaik, 2012). Stratum granulosum is a thin layer that is situated in between stratum lucidum and stratum spinosum (Marks and Miller, 2006); its cells have keratohyalin granules that play an important role in binding of keratin filaments (Wilkes et al., 1973). Stratum spinosum is situated in between stratum granulosum and stratum basale and comprises polyhedral keratinocyte cells which are responsible for the beginning of keratinisation (McGrath et al., 2008; Marks and Miller, 2006). Stratum germinativum, also referred as stratum basale, is the deepest (last) layer of epidermis which is found below the stratum spinosum (Tortora, 2008). Stratum germinativum is characterised as one cell thick epidermal sublayer which contains 5-10 % of melanocytes and is therefore the one responsible for melanin formation (Jimbow et al., 1976). Most of the skin diseases result from hyperproliferation of stratum germinativum (McGrath et al., 2008).

Beneath epidermis is the dermal layer which provides support and nourishment to the epidermis. Dermis is made up of two fibrous materials - collagen and elastin fibres - which consist of collagen and elastin proteins, respectively (Tortora, 2008). The proteins increase the strength and elasticity of dermis. Further, dermis is divided into two sub layers: papillary and reticular. Papillary sub layer

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is described as a thin layer due to loosely attached collagen fibres while reticular sub layer is described as a thick layer due to presence of coarse collagen bundles. In addition to the two sub layers, dermis contains skin appendages such as sweat glands, hair roots, nerve cells and blood and lymph vessels (Katz, 1971). Therefore, dermal layer functions as a temperature regulator, waste remover and stimulator for external stimuli (Kanitakis, 2002).

Subcutaneous fatty tissue (also termed as hypodermis) is situated below the epidermis, and serves as the connector in-between the skin and muscles and bones (Bouwstra et al., 2003). It is composed of elastin fibres and loosely attached connective tissues. Additionally, it contains the macrophages and adipose tissue. Owing to adipose tissue presence, subcutaneous fatty tissue possesses 50 % of body fat that function as a padding and insulator (Madison, 2003).

Skin appendages include the hair follicles, sebaceous glands and sweat glands and are widely distributed from dermal layer up to the stratum corneum sub layer of epidermis (Taylor, 2002). Hair follicles are responsible for hair formation while sebaceous glands produce sebum that gives lubrication to skin and sweat glands regulate the body temperature (Montero-Rievera, 1991).

1.5.2. Skin permeability

Skin permeation is complicated and happens primarily through the transappendageal route and additionally through transepidermal pathway (Scheuplein, 1965). In case of transappendageal pathway, skin appendages like hair follicles, sweat and sebaceous glands are responsible for the skin permeation, whereas transepidermal pathway involves the permeation through stratum corneum (El Maghraby et al., 2008). Compared to transappendageal pathway, transepidermal pathway is a lengthy process and is further subdivided into intercellular and transcellular pathways (Scheuplein, 1965). Skin permeation is impacted upon by the size of the molecule, the physicochemical attributes of the penetrating drug and nanocarriers, the skin condition and the application site (Baroli, 2010)

Skin permeability behaviour of a drug can be studied by several *in vivo* techniques, such as tape or skin stripping. However *in vivo* permeation experimentation has shown enormous difficulty in case of transdermal drug delivery (Williams, 2003). As a result, *in vitro* techniques, such as Franz diffusion cells (Franz, 1975), have recently generated considerable research interest (Williams, 2003). Therefore one of the objective of this study is to conduct the Franz cell study on permeation of 4'-hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) entrapped-interdigitation fusion vesicles (IFV).

1.6. Franz diffusion cell (FDC)

Franz diffusion cell is a hand blown diffusion cell frequently used for permeation assay. This technique was invented by Franz, T.M (Franz, 1978) while conducting studies on percutaneous absorption of organic compounds. Franz diffusion cell is composed of two borosilicate glass chambers that are separated by an excised skin membrane (Fig 1.10).



Fig 1.10: Franz cell apparatus [taken from [http/ www.particlesciences.com](http://www.particlesciences.com)].

The upper compartment is often described as donor compartment. It is also called donor chamber, cell cap or cell top. The bottom compartment is termed as receptor chamber or cell body. The upper compartment is usually positioned on the surface of lower compartment in such way that together they form joint and an excised skin membrane is placed in the middle of the joint with the help of a metallic clamp. Thus, the donor and receptor compartments are separated by the excised skin membrane. Several researchers have used animal skin as the membrane but human skin is the preferred choice (Franz, 1975). In theory, drug formulation is directly applied to the skin

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membrane through donor compartment. The bottom (receptor) compartment possess' receptor medium that is continuously stirred throughout the experiment using a magnetic stirrer at mode 3. The Franz diffusion cells are connected to a closed circuit with circulating water bath at a constant temperature of 37 °C to keep the skin surface at approximately 33 °C. The epithelial skin fragment is equilibrated for 45 min with the receptor flowing while the donor compartment is dehydrated. At regular time intervals, the permeated drug formulation is withdrawn from the receptor compartment using a syringe (1,000 µl) and instantly replaced by an equal volume of fresh receptor medium in order to ensure sink conditions, and to maintain contact between the skin membrane and the receptor medium. The rate and extent of drug permeation via Franz diffusion cell varies significantly depending on the vehicle used. For a formulation scientist, Franz cell study gives an idea about whether the particular drug formulation delivers active agent via the excised skin or not, and to what extent. However, it is very difficult to find out the efficacy of drug formulation using Franz cell analysis and thus, this becomes its limitation (Karande and Mitragotri, 2009). The reason behind failure in determination of efficacy of drug formulation using Franz cell analysis is inadequate penetrability of chemical enhancers. Most of the chemical enhancers that have been studied in the transdermal literature for their capacity to increase transport across skin do not accomplish the desired skin disruption (Karande et al., 2004). They show limited permeation across the stratum corneum themselves and hence their movement is limited to the top few layers of the stratum corneum, as their concentration across the stratum corneum decreases. As a result, these chemicals offer poor transdermal delivery of candidate drug molecules using Franz cell analysis. In order to avoid such problem, here in this project, IFV were used to deliver drugs. This is because liposomes, micelles obtained from phospholipids have been successfully used as permeation enhancers (Cosco et al., 2008). Liposomes composed of lipids such as cholesterol and phospholipids typically act by entrapping drugs in their core and increasing their deposition in the stratum corneum (Fresta and Puglisi, 1996; Mezei and Gulasekharam, 1982; Touitou et al., 1994). Mezei and Gulasekharam (Mezei and Gulasekharam, 1982) already proved that triamcinolone acetonide deposition in skin to be 3- to 6-fold higher when delivered from liposomes as compared to conventional formulations. Skin drug delivery with skin-lipid liposomes was revealed to be more effective than delivery with phospholipid vesicles (Ramon et al., 2005; Kitagawa and Kasamaki, 2006). Phospholipid vesicles do not have an appreciable effect when interacting with the stratum corneum as individual molecules. However, in the form of self-assembled structures as liposomes or micelles, they can easily fuse with the lipid domain of the stratum corneum thereby enhancing partitioning of encapsulated drug as well as disruption of the ordered bilayer structures (Williams and Barry, 1992).

1.7 Aim and Objectives

As mentioned earlier, a few researchers have demonstrated that azo based photosensitive liposomes are beneficial and effective for drug delivery (Morgan et al., 1985; Sandhu et al., 1986). Moreover, Bisby and co-workers (Bisby et al., 2000a) confirmed that azo based photosensitive liposomes can be used for anti-cancer drug delivery. The research from Aston University has also demonstrated that photosensitive liposomes containing Bis-Azo PC are highly effective for tissue engineering (Smith et al., 2007). That means photosensitive liposomes containing Bis-Azo PC can be used for treating other diseases such as psoriasis, vitiligo. Therefore, the central aim of this project was to develop photosensitive liposomes that can be used for the controlled release of drugs through UV irradiation, particularly therapeutic agents for the treatment of skin diseases such as psoriasis, vitiligo. In order to achieve this aim, the following objectives need to be accomplished:

- To synthesize photosensitive lipid i.e. Bis-Azo PC through lipid chemistry method and to characterize the synthesized lipid using NMR, UV, IR and Mass spectrometry.
- To assess the incorporation of photosensitive lipid Bis–Azo PC into various IFV formulations (DPPC:Chol) and the entrapment of a protein marker i.e. bovine serum albumin conjugated with fluorescent isothiocyanate (BSA-FITC). Consequently, to examine the stability of IFV and evaluate the trigger release of protein marker.
- To carry out the trigger release of protein marker using three different UV lamps and to select the most effective lamp that can give higher BSA-FITC release.
- To investigate the incorporation of photosensitive lipid Bis–Azo PC into DPPC: Chol: Bis-Azo PC (16:2:1) based IFV and entrapment of psoralen. Subsequently, to perform the stability studies and morphological analysis of IFV through confocal microscopy; and to estimate trigger release of psoralen.
- To assess skin permeation behaviour of psoralen and psoralen-entrapped photosensitive IFV using Franz cell, and to study the permeation behaviour of psoralen and psoralen-entrapped photosensitive IFV via Leica confocal microscopy.

CHAPTER 2

Synthesis of Bis-Azo Phosphatidylcholine

2.1. Introduction

In recent years much research has focused on photochromism. Photochromism refers to the reversible photo-chemical conversion of chemical compounds to isomers by the absorption of electromagnetic radiation where the two forms possess different absorption spectra (Bouas-Laurent and Desvergne, 2003; Bouas-Laurent and Dürr, 2003; Irie, 2000) . The importance of photochromism in vision is well documented by Wald and co-workers (Wald, 1968). In this report, the researchers evidently indicated that the photoisomerization of 11-cis-retinal to 11-trans-retinal triggers photo-transduction in the retinal photo-receptor cells that ultimately leads to the visual perception. In addition to this, there are numerous other sectors in biochemistry where photochromism can be employed in order to regulate, control and synchronize biological systems. A notable example involves the intonation of ions or drug permeability via liposomes incorporating azobenzene derivatives to undergo photoisomerization upon UV and visible light illumination (Shimomura and Kunitake, 1987). Photoisomerization via UV and visible light irradiation has benefits of supplying a broad range of adjustable parameters such as wavelength, duration and intensity, which can be easily optimized in a non-disturbing manner; in contrast, other photo-chemical transformations using photo-polymerization (Spratt et al., 2003; Bondurant et al., 2001; Miller et al., 1996) or photo-oxidation (Wymer et al., 1998; Collier et al., 2001; Smeds et al., 2001) of membrane lipids can disturb the integrity of bilayer membranes in order to enhance release of ions or drug materials from lipid vesicles. These photochemical processes using photosensitive liposomes have been discussed in detail in the general introduction chapter (see section 1.3.3.1).

Photoisomerization via UV and visible light irradiation can be achieved by incorporating either azobenzene derivatives (Shimomura and Kunitake, 1981), stilbenes (Saltiel et al., 1968) or olefins (Adhikari et al., 2001). Contrary to stilbenes and olefins, whose photoisomerization reaction displays a high solution sensitivity, photoisomerization via azobenzene derivatives has been found to take place in viscous solutions, liquid crystals and solid materials (Bondurant et al., 2001; Bortolus and Monti, 1987; Haberfield, 1987; Monti et al., 1982; Rau and Lueddecke, 1982; Shinkai et al., 1982; Yamamoto, 1986). There is extensive literature available on the thermal and photoisomerization reactions using azobenzene derivatives (Siampiringue et al., 1987; Jones and Hammond, 1965; Hammond et al., 1964). Therefore, photoisomerization using azobenzene derivatives has received considerable interest and these derivatives are used as photoresponsive “triggers” for control of membrane permeability (Okahata et al., 1986).

The photoisomerization of azobenzene and its derivatives occurs due to the presence of the azo group ($-N=N-$) in conjugation with two phenyl substituents. Even the UV/visible spectroscopic features of these aromatic compounds also depend on the presence of aromatic azo group. The presence of double bonds between the two nitrogen atoms gives rise to two different isomers i.e. E-

or Z- configuration. Therefore in the presence of UV light (about 320 nm), the more stable trans isomer of azobenzene (E) is transformed into the less stable cis isomer of azobenzene (Z), while in the presence of visible light (about 430 nm) the cis isomer is transformed into trans isomer (Fig 2.1).

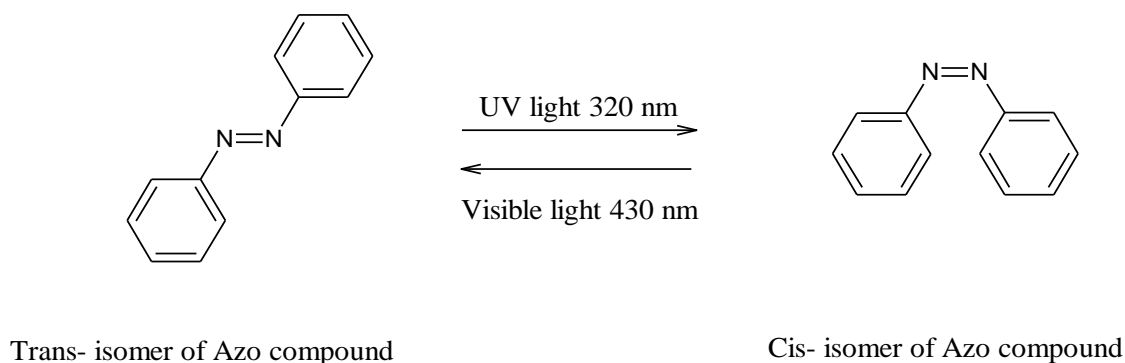


Fig 2.1: Trans-Cis isomerization of azo-compound in the presence of light.

This cis-trans isomerization is supplemented by a huge geometrical and structural change. As a result, the para carbon atoms in the trans-isomer of azobenzene are at a distance of about 9.0 Å whereas in the cis-isomer they are only at a distance of about 5.5 Å. Even the retrenchment is higher in the cis form of azobenzene. Morgan and co-workers (Morgan et al., 1987b) have proven that the trans form of azobenzene has a virtually planar and linear structure with no dipole moment, whereas the cis form of azobenzene possesses dipole moment and the two aromatic ring of this isomer are out of plane due to orientation of the long axes of the aromatic ring at an angle of 56°. This research group also documented that photochemical decomposition is not major issue in case of azobenzene and its derivatives. Most importantly, azobenzenes are attractive because the isomerization is reversible and effective in terms of drug delivery on demand. All these unique features make azobenzene and its derivatives suitable candidates for the liposomal photo-triggers in terms of photosensitive liposomes. To date several different photo-triggering approaches incorporating azobenzene and its derivatives as the photo-control switch have been tried (Fig 2.2). Kano and colleagues (Kano et al., 1981) carried out the initial experiments in 1981 in order to investigate the membrane permeability of liposomes using a single chain amphiphilic azobenzene derivatives i.e. Azo-PC. They effectively incorporated C₂-Azo-C₁₂ and C₄-Azo-C₁₂ into the bilayer membrane of dipalmitoyl phosphatidylcholine (DPPC) liposomes. However, Shimomura and co-workers (Shimomura and Kunitake, 1981) demonstrated that the single chain amphiphilic azobenzene derivatives stimulate the fusion and phase separation fusion process in bilayer membranes. Later on Morgan and his research group (Morgan et al., 1985) introduced double chain azobenzene derivatives i.e. Bis-Azo Phosphatidylcholine (Bis-Azo PC). UV irradiation causes trans to cis isomerization of both azobenzene fragments that lead to more disturbances in the bilayer than observed with single chain of azobenzene derivative i.e. Azo-PC.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

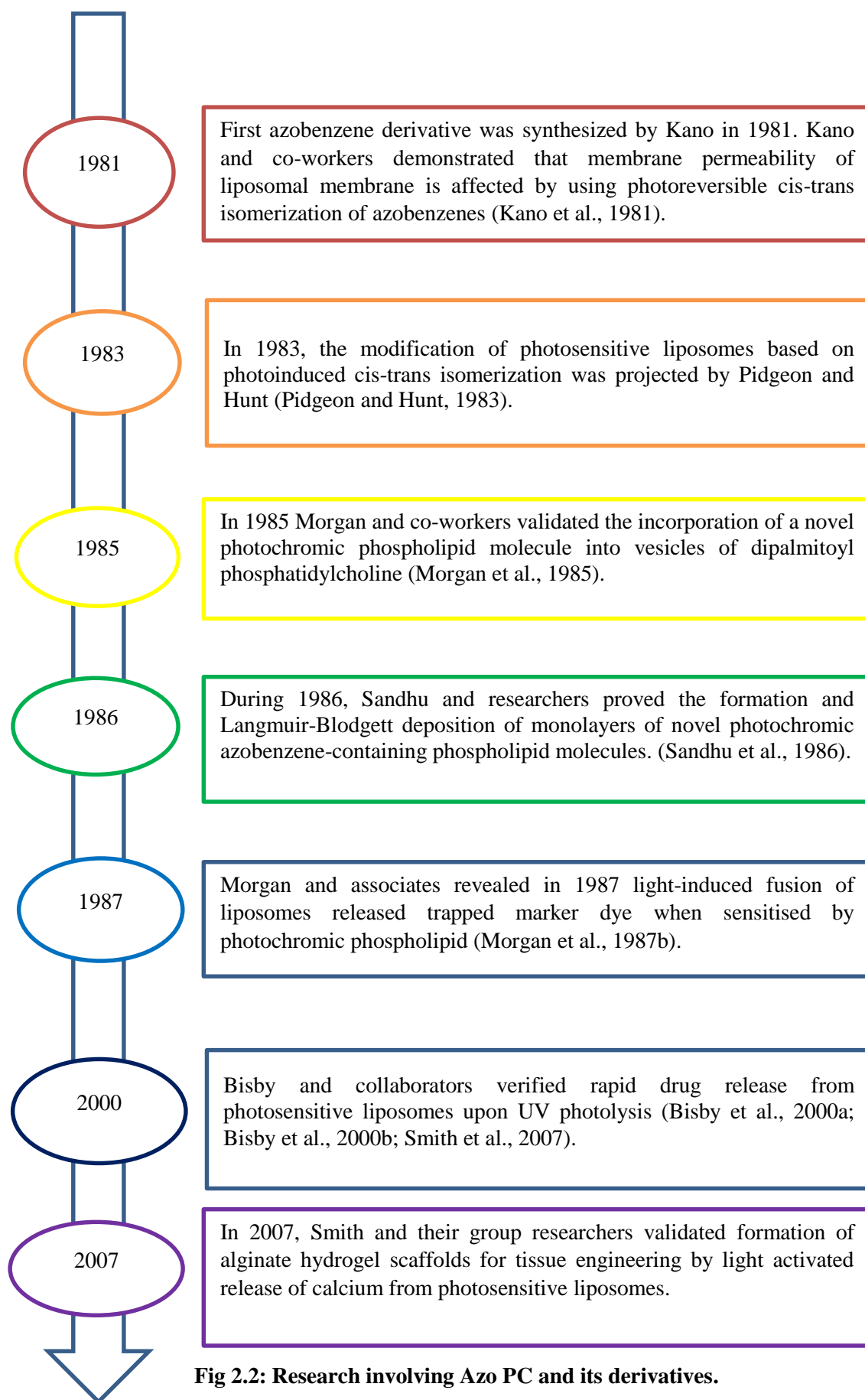


Fig 2.2: Research involving Azo PC and its derivatives.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

The liposomes containing Bis-Azo PC led to rapid release of entrapped drug upon UV light illumination. A few minutes of UV irradiation cause complete release of entrapped material which is not beneficial to the sustained or multipulsatile drug release. As a result, during 2000, Bisby and research group (Bisby et al., 2000a; Bisby et al., 2000b) introduced varying amount of Bis-Azo PC and cholesterol in order to minimize perturbation and spontaneous release to bilayer membranes. Similar research also indicated that liposome of DPPC containing Bis-Azo PC (6 mol %) and cholesterol (up to 15 mol %) showed no significant leakage of calcein for at least 8 weeks, while liposomes containing higher concentrations of Bis-Azo PC (e.g. 10 mol %) are stable overnight, but release their contents during a long term storage. Thus, by using cholesterol, liposomes containing Bis-Azo PC offer a suitable means to 'cage' water soluble compounds (e.g. calcium and copper) and drugs such as methotrexate (Morgan et al., 1991). For that reason, this system is beneficial in terms of drug delivery and photodynamic therapy.

In 2007, Smith et al. (Smith et al., 2007) used Bis-Azo PC incorporated liposomes with the purpose of trigger gelation of an alginate solution by releasing calcium chloride upon irradiation with 385 nm light for 1 min. This strategy plays a key role in tissue engineering.

2.2. Aim and Objectives:

The aim of this part of the project was to synthesize and characterise Bis-Azo Phosphatidylcholine with the method reported previously by Sandhu and co-workers (Sandhu et al., 1986) with some modifications. In order to achieve this, specific objectives were:

- Preparation and characterisation of 4-(n-butyl) nitrosobenzene.
- Preparation and characterisation of Azo acid compound.
- Synthesis and characterisation of Bis-Azo Phosphatidylcholine.

2.3. Materials and Experimental Methods

2.3.1 Chemicals

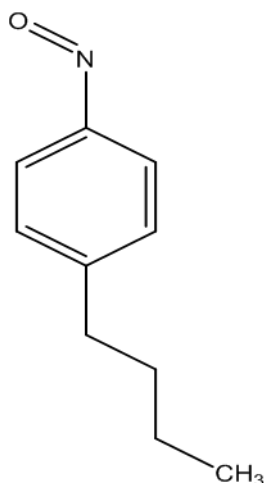
Unless otherwise noted all reactions were executed in oven dried glassware. Chemicals unless specified, were sourced from Sigma (Sigma-Aldrich, UK) or Fisher (Fisher Scientific, UK) without further purification. All aqueous solutions were prepared with double distilled deionised water. Pivaloyl chloride, 4-(4-aminophenyl) butanoic acid and 4 - butylaniline were purchased from Alfa Aesar, UK. Potassium peroxymonosulphate was obtained from Acros Organics, UK. Aluminium silica gel 60 F254 plates (Merck, Germany) were used for thin layer chromatography (TLC). B.D.H silica gel (MFC) (Merck, Germany) Kieselgel Art 7736 was used for flash column chromatography.

2.3.2 Instrumentation

NMR spectra were recorded on a Bruker AC- 250 instrument (250MHz for ^1H ; 62.9 MHz for ^{13}C) spectrometer, at 23 °C, 64 scans at an acquisition time of 3 seconds. The samples were dissolved in DMSO- d_6 and referenced at $\delta\text{DMSO} = 2.50$ ppm unless otherwise stated. ^1H and ^{13}C NMR chemical shifts are reported as parts per million (ppm) relative to TMS (0.00 ppm). A Mattson 3000 FTIR spectrophotometer was used to obtain infrared spectra by using either KBr disc method or cast film technique using NaCl cells or a Thermo Scientific Nicolet is5 with iD5 ATR connection as a solid sample. The synthesized materials were poured into quartz cuvette (pathlength 1 cm) for optical absorption using a Cary 1 E UV–Vis spectrophotometer. A Hewlett-Packard 5989B quadrupole instrument connected to an electrospray 59987A unit with an atmospheric pressure chemical ionisation (APCI) accessory and automatic injection using a Hewlett-Packard 1100 series autosampler was used to obtain APCI mass spectrum and Electrospray ionisation (ES) for some mass spectra or a Waters 2795 LC/MS using Mass lynx software for others. Melting points were measured on a Reichert-Jung Thermo Galen hot stage microscope and corrected by calibrating against a compound of known melting point.

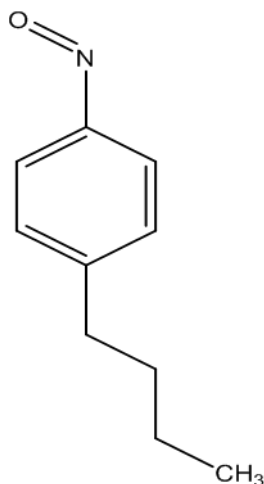
2.3.3. Experimentation

2.3.3.1 Preparation of 4-(*n*-butyl) nitrosobenzene- method 1:



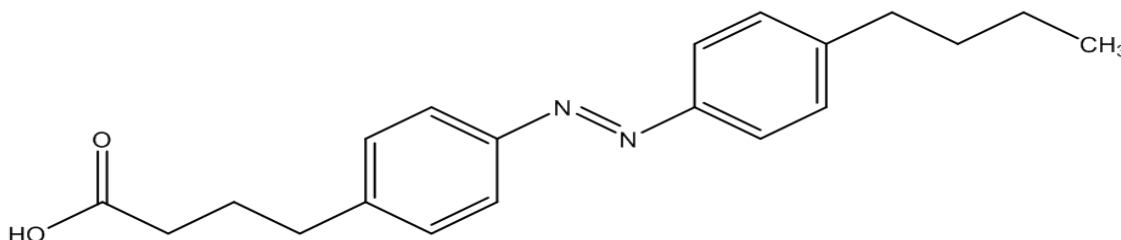
This method was adapted from Kennedy and Stock (Kennedy and Stock, 1960) with some modifications. 4- Butylaniline (2.11 mL, 0.013 mol) was added into the RBF (150 mL) containing 10 mL of diethyl ether. Potassium peroxymonosulphate (5 g, 0.033 mol) was dissolved in 30 mL of water in a beaker and this solution was added rapidly to the above solution. The mixture was allowed to cool to room temperature and the solution was made basic by the addition of sodium carbonate (0.5 g) while monitoring the pH. The ether layer and a yellow solid which had precipitated were separated from the aqueous phase of the mixture. Then, the aqueous solution was extracted continuously with diethyl ether for a period of 30 min. The diethyl ether extractions were combined, dried over anhydrous magnesium sulphate, filtered and evaporated to dryness. The crude 4-(*n*-butyl) nitrosobenzene thus obtained was recrystallized from ethanol, giving a yellow product (1.65 g, 78 %). *R_f* (chloroform): 0.43; melting point: 116 °C; ¹H NMR (250 MHz, DMSO-*d*₆) δ: 8.16 (d, 2H, aromatic), 7.31 (d, 2H, aromatic), 2.66 (m, 2H, -CH₂CH₂CH₂CH₃), 1.60 (m, 2H, CH₂CH₂CH₂CH₃), 1.30 (m, 2H, CH₂CH₂CH₂CH₃), 0.91 (m, 2H, CH₂CH₂CH₂CH₃); ¹³C NMR (62.9 MHz, DMSO-*d*₆) δ: 146.50, 142.22, 129.22, 122.19, 36.02, 30.94, 22.28, 13.95.

2.3.3.2. Preparation of 4-(n-butyl) nitrosobenzene - method 2:



This method was adapted from Okazaki et al 1969 (Okazaki et al., 1969) with some modifications. To a RBF, peroxybenzoic acid (1.44 g, 0.01 moles) was added to 10 mL of ice-cooled dichloromethane (solution A). In a separate flask, 4-butylaniline (0.5 mL, 0.003 moles) was added to 10 mL of ice-cooled dichloromethane (solution B). Then, solution B was added dropwise into the RBF containing solution A with constant stirring at 0 °C. The reaction was kept overnight at 0 °C, after which the reaction mixture was shaken with aqueous sodium carbonate in order to remove benzoic acid. The organic layer was dried over anhydrous magnesium sulphate and then the solvent was evaporated under reduced pressure on the rotary evaporator. The crude product was recrystallized from ethanol, giving the final compound as a yellow coloured product (0.685 g, 30 %). R_f (chloroform): 0.43; melting point: 116 °C; ¹H NMR (250 MHz, DMSO-d₆) δ: 8.16 (d, 2H, aromatic), 7.31 (d, 2H, aromatic), 2.66 (m, 2H, -CH₂CH₂CH₂CH₃), 1.60 (m, 2H, CH₂CH₂CH₂CH₃), 1.30 (m, 2H, CH₂CH₂CH₂CH₃), 0.91 (m, 3H, CH₂CH₂CH₂CH₃); ¹³C NMR (62.9 MHz, DMSO-d₆) δ: 150.70, 146.50, 129.70, 122.64, 36.02, 30.94, 22.28, 13.95.

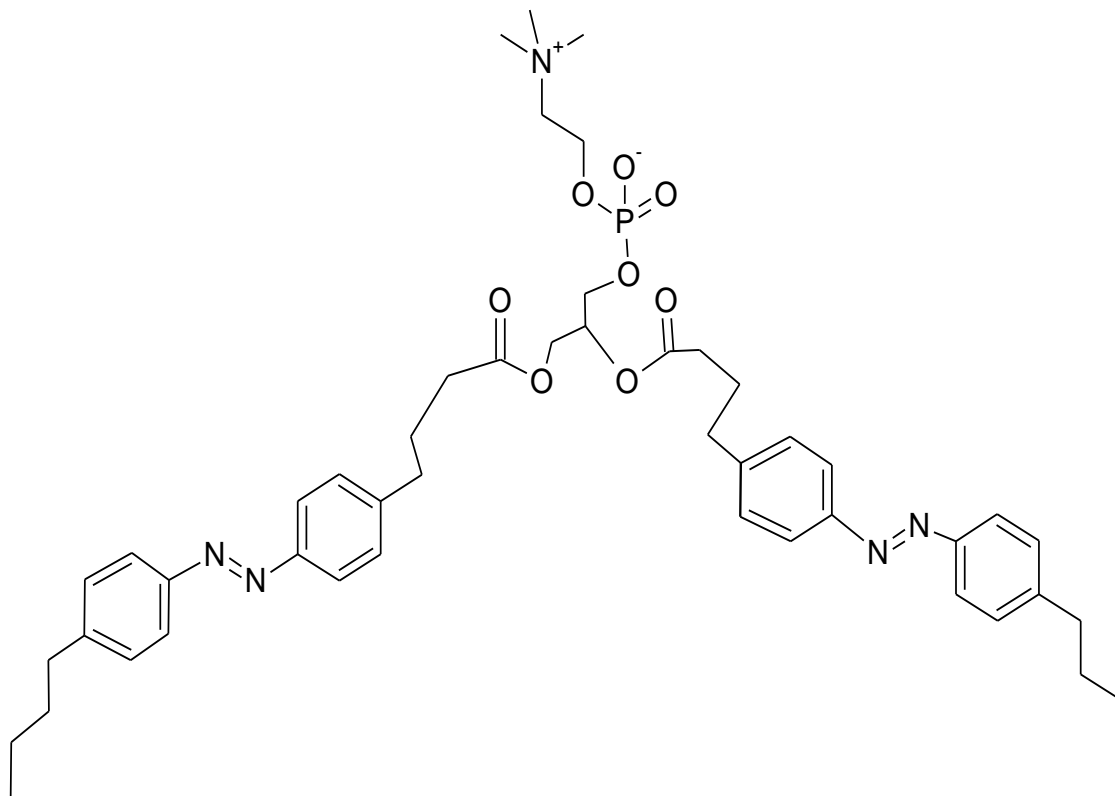
2.3.3.3. General procedure for the preparation of 4-{4-[(4-butylphenyl) diazenyl] phenyl} butanoic acid



The synthesis of this compound was adapted from Morgan and co-workers (Morgan et al., 1987b; Morgan et al., 1985; Sandhu et al., 1986).

4-(n-butyl) nitrosobenzene (1.3 g, 0.008 moles) and 4-(4-aminophenyl) butyric acid (1 g, 0.005 mols) were dissolved in 6 mL of glacial acetic acid at room temperature, and the whole mixture was allowed to stand for several hours. At this stage, crystalline 4-(n-butyl)-4' (3-carboxypropyl) azobenzene separated out and was filtered off. The product was dissolved in chloroform, washed with 10% aqueous sodium bicarbonate and dried over anhydrous magnesium sulphate. Purification of azo acid compound was done by column chromatography eluting with chloroform/petroleum ether (50:50, v/v). The final product was obtained as a brownish yellow solid (1.004 g, 62 %). R_f (Chloroform: petroleum ether 1:1) = 0.5; melting point: 35-40 °C; ^1H NMR (250 MHz, DMSO- d_6) δ : 7.80 (d, 2H, aromatic), 7.80 (d, 2H, aromatic), 7.32 (d, 2H, aromatic), 7.32 (d, 2H, aromatic), 2.65 (m, 2H, $\text{HOOCCH}_2\text{CH}_2\text{CH}_2$), 2.54 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 2.33 (m, 2H, $\text{HOOCCH}_2\text{CH}_2\text{CH}_2$), 2.18 (t, 2H, $\text{HOOCCH}_2\text{CH}_2\text{CH}_2$), 1.68 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.36 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 0.91 (t, 3H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$); ^{13}C NMR (62.9 MHz, DMSO- d_6) δ : 178.45, 150.99, 146.15, 129.84, 129.24, 122.70, 122.08, 37.73, 34.09, 32.47, 30.94, 26.99, 22.29, 13.94; MS: calculated for $[\text{C}_{20}\text{H}_{23}\text{N}_2\text{O}_2]$ 324.18, found 323.17 (M-H); IR (near) cm^{-1} = 3419, 2954, 2928, 2856, 1602, 1180, 1465, 839, 548; λ_{max} - 334 nm.

2.3.3.4. Synthesis of Bis-Azo Phosphatidylcholine



To a stirred suspension of 4-{4-[(4-butylphenyl)diazenyl]}butanoic acid (0.0091 g, 0.002 moles) in 2 mL of dry methylene chloride, redistilled triethylamine (4.5 μ L) and pivaloyl chloride (15 μ L) were added. The reaction was incubated for 120 minutes at 25 °C with continuous stirring. The reaction was monitored by TLC to confirm the formation of mixed anhydride. The compound was vacuum dried for 120 minutes at 25 °C (Morgan et al., 1982).

Next, sn-Glycero-3-phosphorylcholine (0.257 g, 0.001 moles) was suspended in 20 mL of freshly distilled anhydrous chloroform followed by the addition of 4-(N, N-dimethylamino) pyridine (DMAP) (0.244 g, 0.002 moles) and mixed anhydride (0.810 g, 0.003 moles) synthesised. The reaction vessel was flushed with nitrogen, the reaction flask tightly sealed, and the mixture was stirred at ambient temperature. Protection from light was necessary because of the sensitivity of anhydrous ethanol-free chloroform. After completion of the reaction (35-40 hr), the solvent was removed under reduced pressure and the residue was treated with 20 mL of methanol /chloroform/water (5:4:1, v/v/v). After filtration through a sintered –glass funnel, the solution was

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

evaporated and the desired product was purified by column chromatography eluting with chloroform/methanol/water (65:25:10, v/v/v). In order to remove any silica gel particles, the phospholipids were filtered through a sintered –glass funnel as a solution in methylene chloride and dried to obtain final product as a brownish red solid (0.0115 g, 70 %) yield (Radhakrishnan et al., 1981). R_f (Chloroform) = 0.45; ¹H NMR (250 MHz, DMSO-d₆) δ: 7.84 (d, 1H, aromatic), 7.84 (d, 1H, aromatic), 7.27 (d, 1H, aromatic), 7.27 (d, 1H, aromatic), 6.55 (m, 1H, OCH₂OCHCH₂), 4.22 (d, 2H, OCH₂OCHCH₂), 3.66 (d, 2H, OCH₂OCHCH₂), 3.34(s, 1H, OOOPOCH₂CH₂N(CH₃)₃), 3.20 (m, 2H, OOOPOCH₂CH₂N(CH₃)₃), 3.07(s, 9H, N(CH₃)₃), 2.58 (m, 4H, CH₂CH₂CH₂CH₃), 2.37 (m, 4H, OOCCH₂CH₂CH₂), 2.18 (t, 4H, OOCCH₂CH₂CH₂), 1.92 (s, 4H, OOCCH₂CH₂CH₂), 1.65 (m, 4H, CH₂CH₂CH₂CH₃), 1.25 (s, 4H, CH₂CH₂CH₂CH₃), 0.94 (t, 6H, CH₂CH₂CH₂CH₃); ¹³C NMR (62.9 MHz, DMSO-d₆) δ: 172.44, 155.12, 150.12, 149.68, 146.58, 129.17, 129.07, 123.15, 122.78, 79.70, 70.90, 66.03, 62.96, 58.57, 56.92, 39.34, 35.57, 33.47, 30.94, 27.70, 22.34, 13.95; MS: calculated for [C₄₈H₆₄N₅O₈P] is (870.02), found mass: (870.50); IR (near) cm⁻¹: 3390, 2958, 2927, 2343, 2362, 1640, 1612, 1534, 1380, 1226, 1066, 946, 817, 663; λ_{max} :- 256 nm, 332nm.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

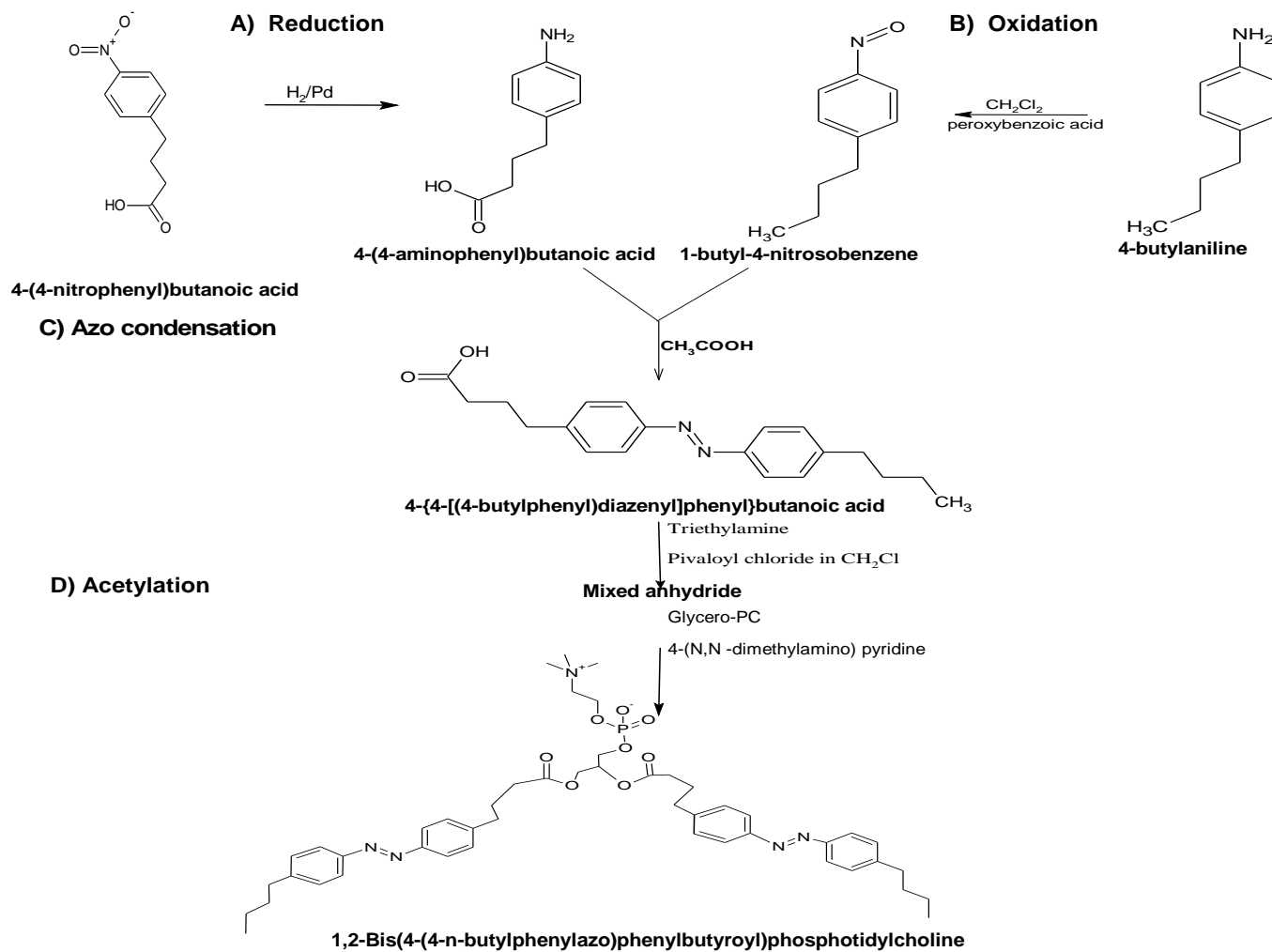


Fig. 2.3: General procedure involved in the synthesis of Bis-Azo Phosphatidylcholine.

2.4. Discussion

The synthesis of Bis-Azo PC is not an easy process, though it looks very straightforward from Fig 2.3. It involved the three major steps: 1) synthesis of Nitrosobenzene, 2) synthesis of azo acid and 3) synthesis of Bis-Azo Phosphatidylcholine. The characterisation of these compounds has not been reported previously. However, few articles noted only NMR results of final compound i.e. Bis-Azo Phosphatidylcholine.

2.4.1. Synthesis of 4-(n-butyl) nitrosobenzene

4-(n-butyl) nitrosobenzene was synthesized by two methods. In both methods 4-butylniline was used as the starting material but with different oxidizing agents. The first method gave prominent result and better yield compared with the second method.

In the first method, the synthesis of 4-(n-butyl) nitrosobenzene involved the oxidation of 4-butylniline with potassium peroxymonosulphate in ether (Fig 2.4a and b). This was based on a method reported previously (Kennedy and Stock, 1960) with some modifications. As this was exothermic reaction, whole process was carried in an ice bath. During the reaction, ether was used to dissolve the starting material and water was used to dissolve the oxidizing agent, potassium peroxymonosulphate.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

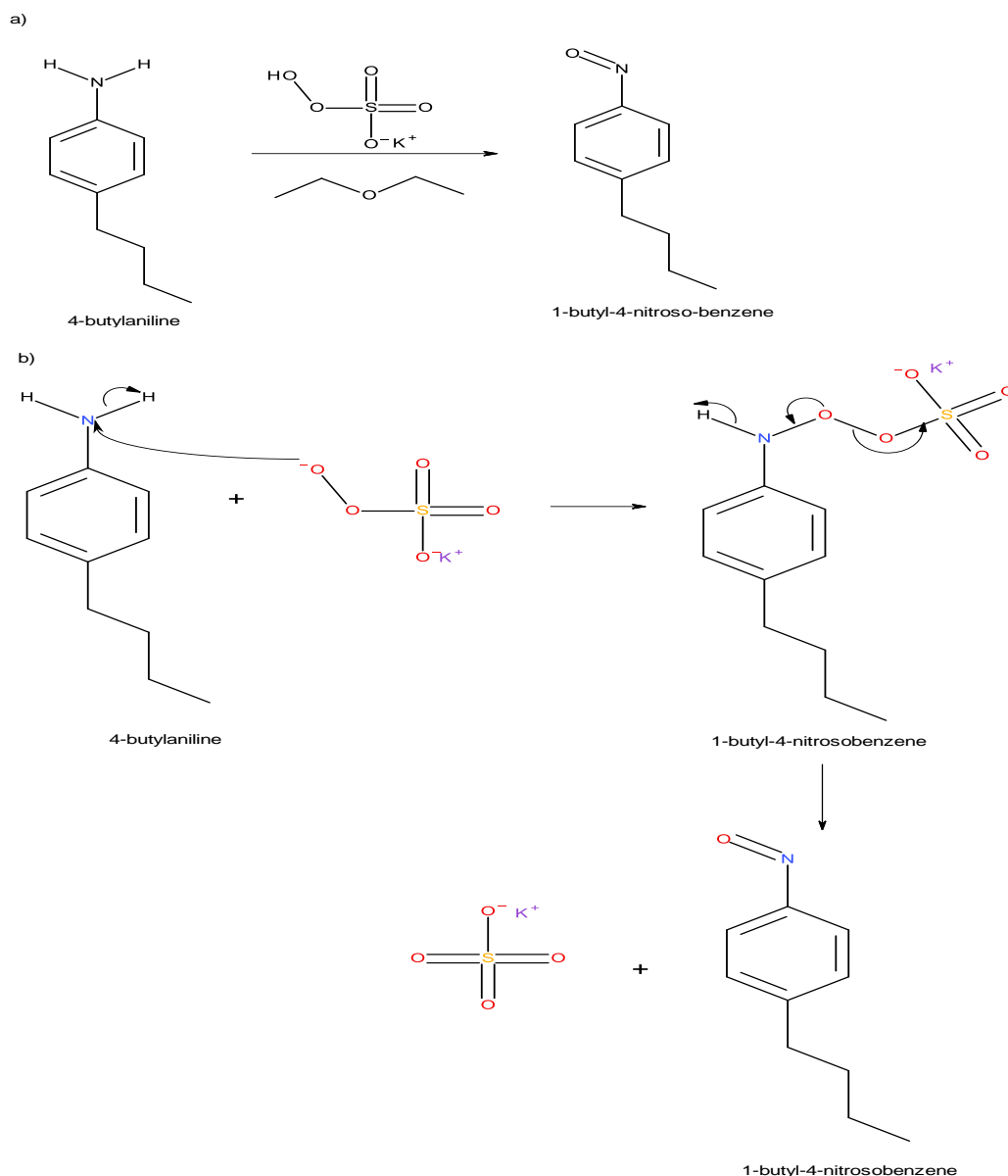


Fig 2.4: a) General equation for the synthesis of 4-(n-butyl) nitrosobenzene, and b) mechanism behind this synthesis.

The whole reaction was completed within five hours. Furthermore, purity of product was determined by chromatography and melting point study.

Thin layer chromatography (TLC) was conducted to determine the purity of 4-(n-butyl) nitrosobenzene. TLC results obtained from the resulting reaction mixture clearly indicated two spots (Fig 2.5 a) on silica plate. It could be inferred that the product was contaminated with some amount of starting material. Therefore, 4-(n-butyl) nitrosobenzene was purified by recrystallization. Several recrystallizations were attempted with various solvents including ether (the solvent which was used to dissolve the starting material) however, with very limited success. At last, 4-(n-butyl)

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

nitrosobenzene was recrystallized by ethanol. TLC analysis was conducted again in order to verify the purity of the product and the results showed a single spot (Fig 2.5 b). As can be seen from Fig 2.5, analytical TLC on a silica gel plate showed that the starting material and the resulting mixture travel on silica adsorbent at different rates, when chloroform was used as the developing solvent.

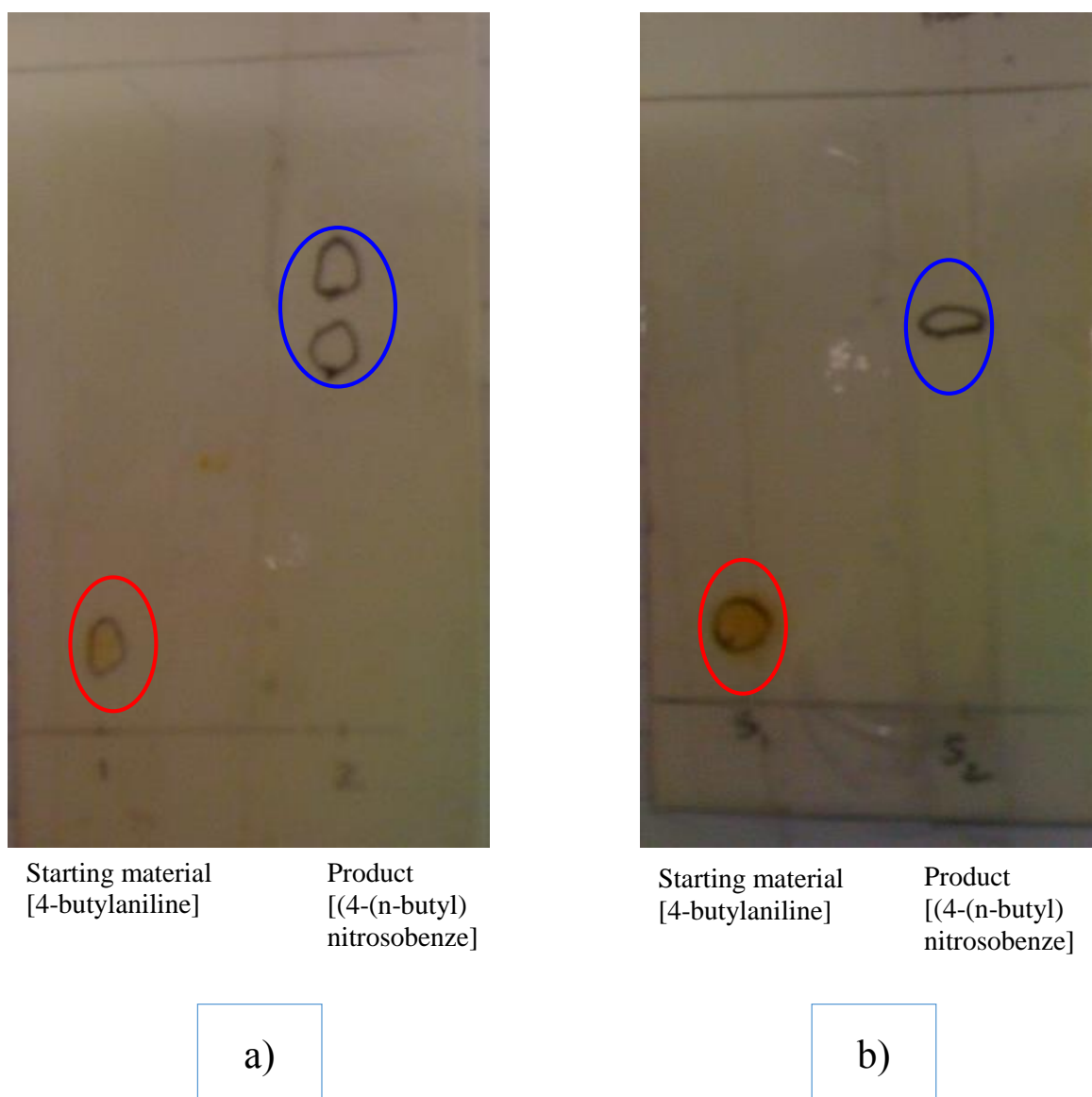


Fig 2.5: TLC analysis of 4-(n-butyl) nitrosobenzene before (a) and after (b) recrystallization with ethanol.

The purified product was characterized by nuclear magnetic resonance (NMR), Ultraviolet (UV) spectroscopy (see Section 2.3.3.2) and its melting point was determined with Gallenkamp Variable Heater Melting Point Apparatus and found to be 116 °C.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

Initially, while performing the nitrosobenzene synthesis, an additional undesired white powder was obtained, which could not be removed by recrystallization. Several recrystallization attempts were carried out in various solvents such as ether, chloroform, but without success. It was presumed that the undesired product might be due to the rapid addition of the oxidizing agent, potassium peroxymonosulphate. A dropwise addition of oxidizing agent in the solution reduced the formation of the undesirable white product.

The second approach (see section 2.3.1 method- 2) to synthesize 4-(*n*-butyl) nitrobenzene using 4-butylaniline with peroxybenzoic acid in presence of dichloromethane (Fig.2.6) was not to encouraging, because the product yield was very low (30 %). In addition, cooling and stirring for overnight was time consuming. Overall, second method did not give satisfactory results. . Therefore method 1 was used to prepare 1-butyl-4-nitrosobenzene.

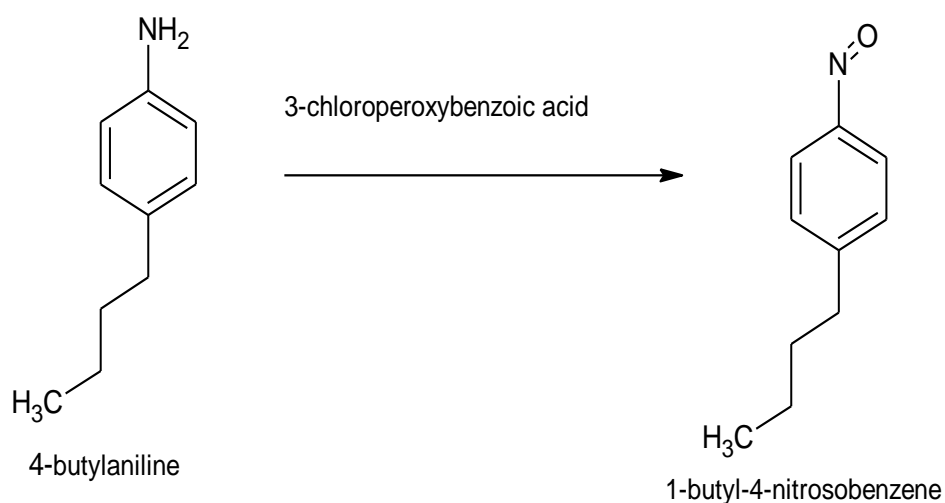


Fig 2.6: General equation for the synthesis of 1-butyl-4-nitrosobenzene by second method.

2.4.2. Synthesis of 4-{4-[(4-butylphenyl) diazenyl] phenyl} butanoic acid

Azo Acid was successfully synthesized by the azo condensation reaction, in which, the equimolar amount of 4-(*n*-butyl) nitrosobenzene and 4-(*p*-aminophenyl) butyric acid was used (Fig 2.7).

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

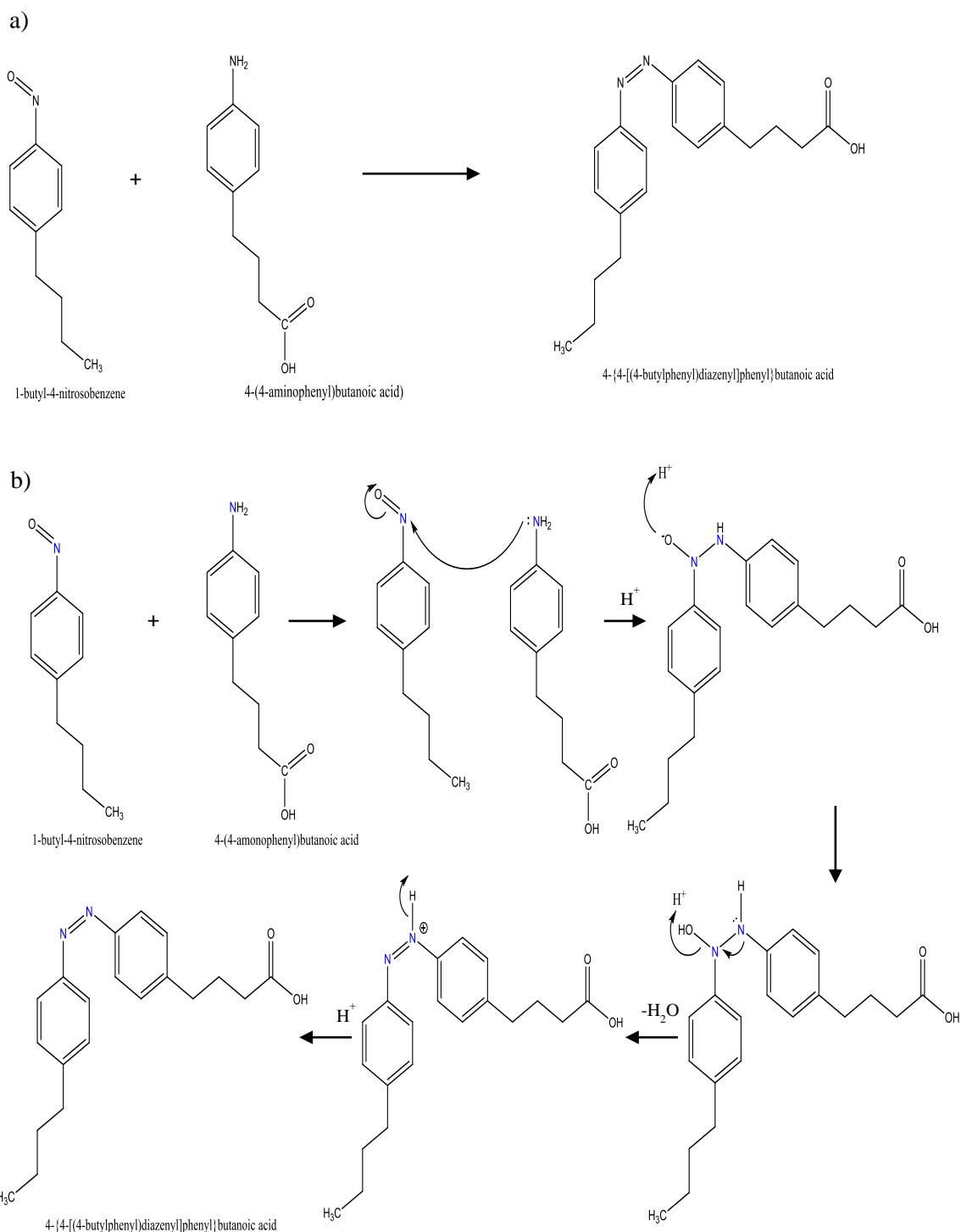


Fig 2.7: a) General equation for the preparation of 4-{4-[(4-butylphenyl) diazenyl] phenyl} butanoic acid, and b) Mechanism behind this reaction.

Though reaction was very simple and straightforward, but the product yield was very low and impure. Therefore, resultant product was separated by flash column chromatography (FCC).

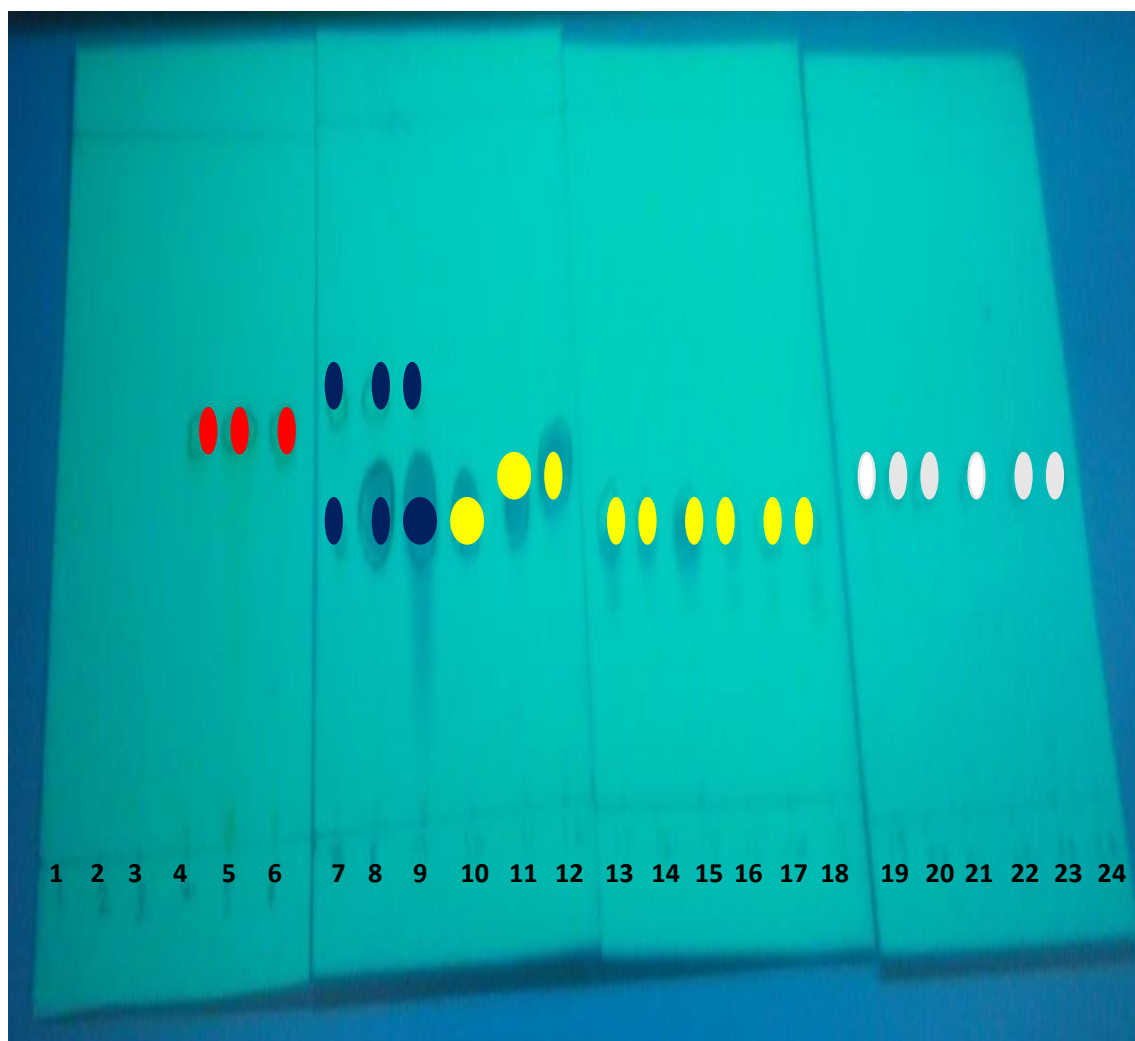


Fig. 2.8: TLC analysis of 4-{4-[(4-butylphenyl) diazenyl] phenyl} butanoic acid.

Details of spots detected in above figure are as follows, red coloured spots from 4 to 6: fractions of desired compound, dark blue coloured spots from 7 to 9: contaminated with desired and undesired compound, yellow coloured spots 10 to 18: undesired compound and tan coloured spots 19 to 24: polar impurities. Elution solvent was chloroform/petroleum ether (50:50, v/v).

Fig. 2.8 above illustrates a silica-gel plate, onto which were spotted samples, with the purpose of analysing and preparing azo acid. Column chromatography was performed using chloroform/petroleum ether (50:50, v/v) as eluting solvent, and the fractions collected in numbered test tubes (1-24). During this Azo acid separation, the mobile phase in the system migrate through the stationary phase i.e. silica gel with the help of isocratic pump; therefore the different components of crude sample are eluted at different rates due to their migration at different speeds. It has been reported that the elution of components was dependent on their polarities, the nature of the stationary phase (adsorbent), and the solvent used for elution (mobile phase) (Jack et al., 2002). As indicated in Fig.2.8, from left to right, the pure compound of interest Azo acid was

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collected in tubes 4-6, meanwhile impure samples collected in tubes 7-9. Moreover, undesirable compounds were collected in tubes 10-18 and polar samples collected in tubes 19-24. In theory, Column chromatography removes any polar impurities, intermediate impurities and unreacted 4-(*n*-butyl) nitrosobenzene.

Based on the TLC results, it could be speculated that an eluting solvent which gives an R_f value of 0.5 for the most polar compound gives a better Azo acid separation from the crude samples

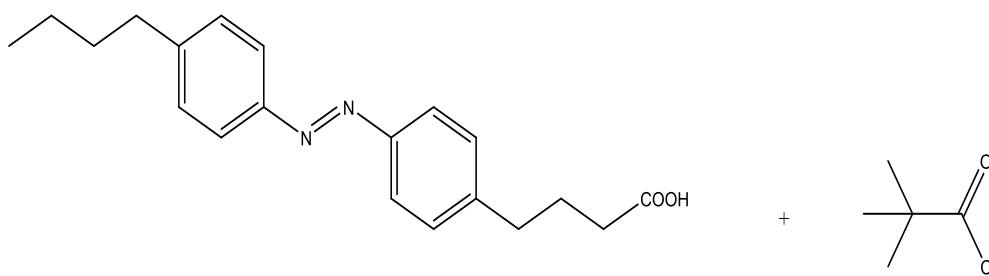
Product from test tubes 4-6 showed desired product i.e. 4-{4-[(4-butylphenyl) diazenyl] phenyl} butanoic acid which was characterized by NMR, UV, Mass spectrometry and Infra-red (IR) spectroscopy (see Section 2.3.3.3).

2.4.3 Synthesis of Bis-Azo Phosphatidylcholine

Bis-Azo Phosphatidylcholine was synthesized by azo acid and L-alpha glycerophosphatidylcholine (Fig 2.9). Reaction mechanism involved two steps: acetylation reaction and attachment of phosphatidylcholine. Synthesis and purity of Bis-Azo Phosphatidylcholine depends upon the azo acid anhydride formation and the phosphatidylcholine attachment (Fig 2.10). The stereochemistry of Bis-Azo pc in the presence of light is shown in Fig 2.11. The resultant product was characterized by NMR, UV and IR.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

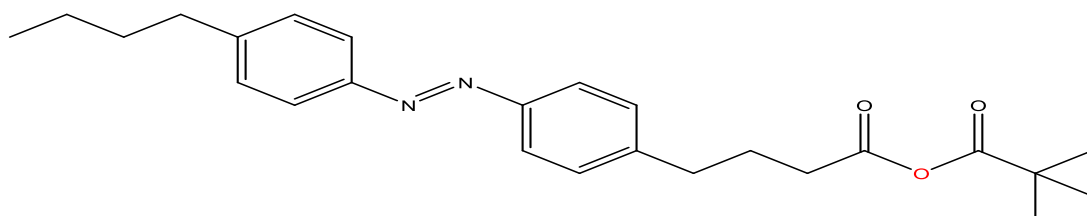
Step: 1



Trans- isomer of 4-{4-[(4-butylphenyl) diazenyl] phenyl} butanoic acid

Pivaloyl chloride

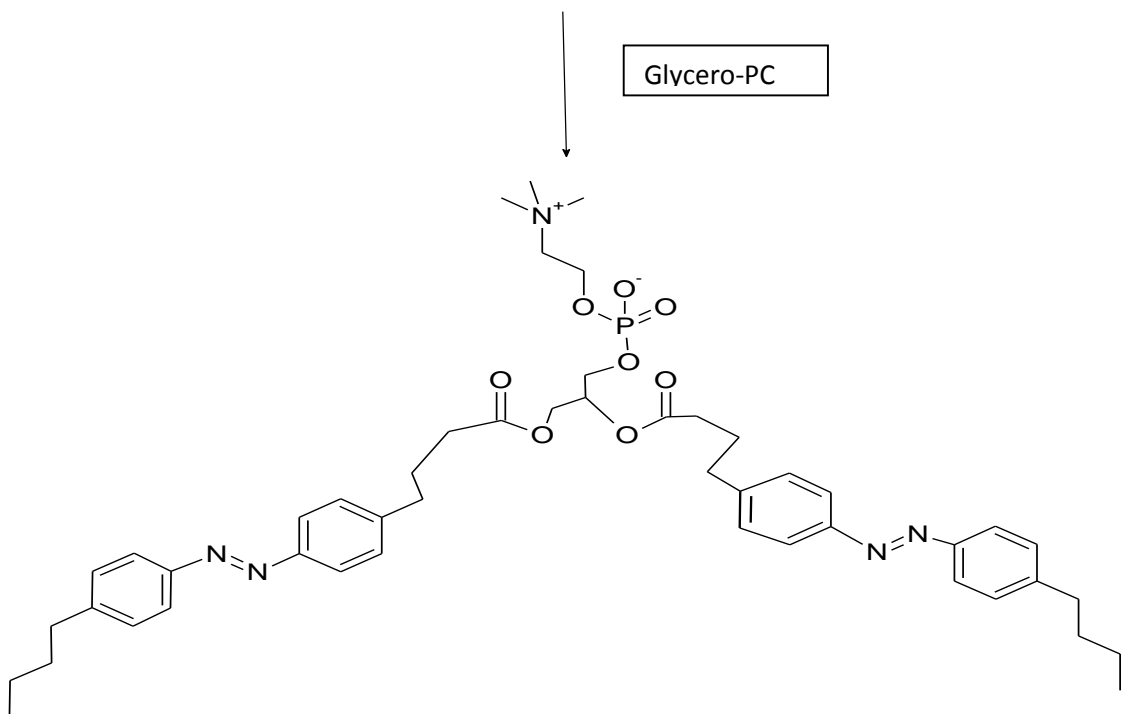
4-(N, N-dimethylamino) pyridine (DMAP)
 CH_2Cl_2



Step: 2

Trans- isomer of Mixed Anhydride

Glycero-PC



Trans- isomer of Bis-Azo Phosphatidylcholine

Fig 2.9: Preparation of Bis-Azo Phosphatidylcholine.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

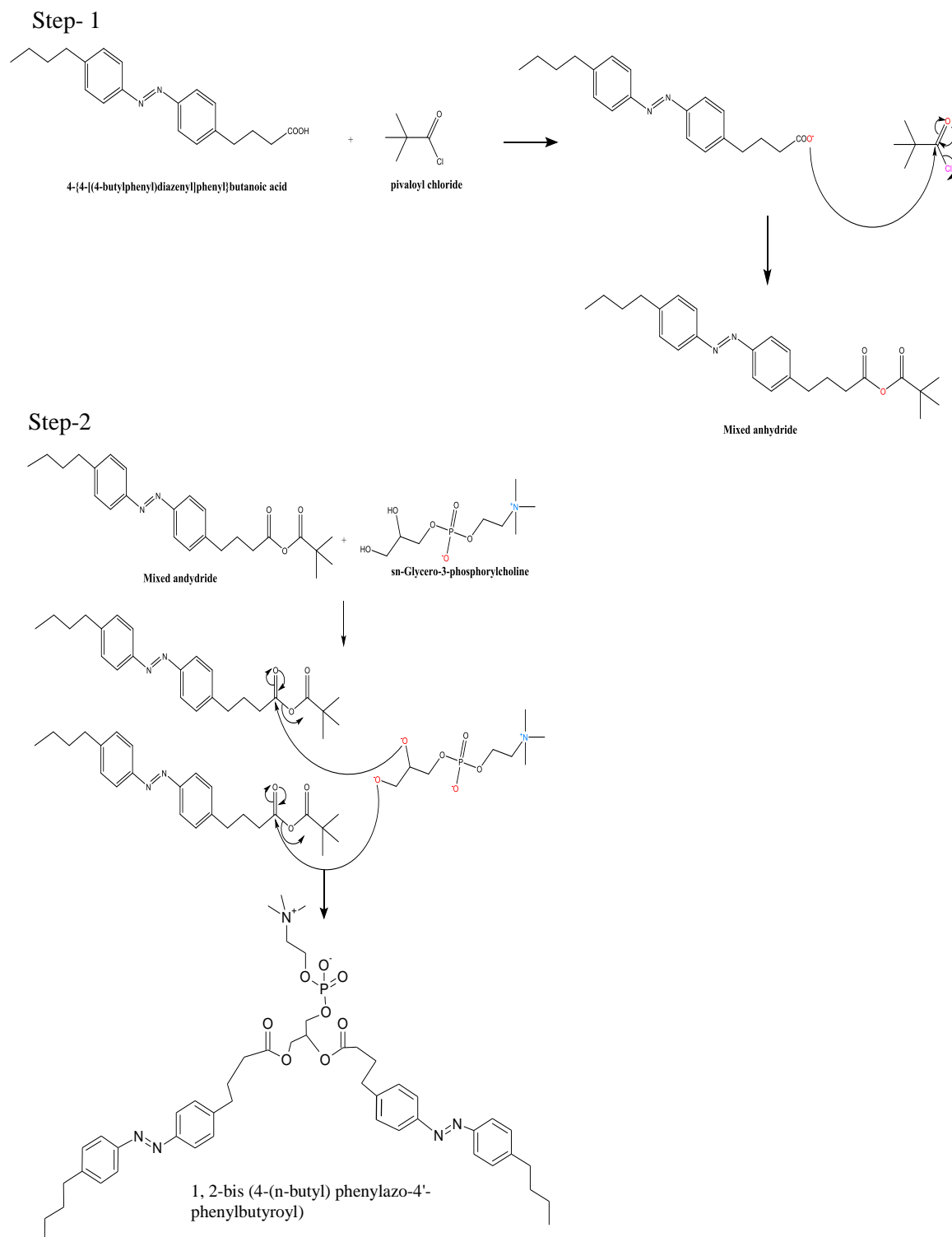
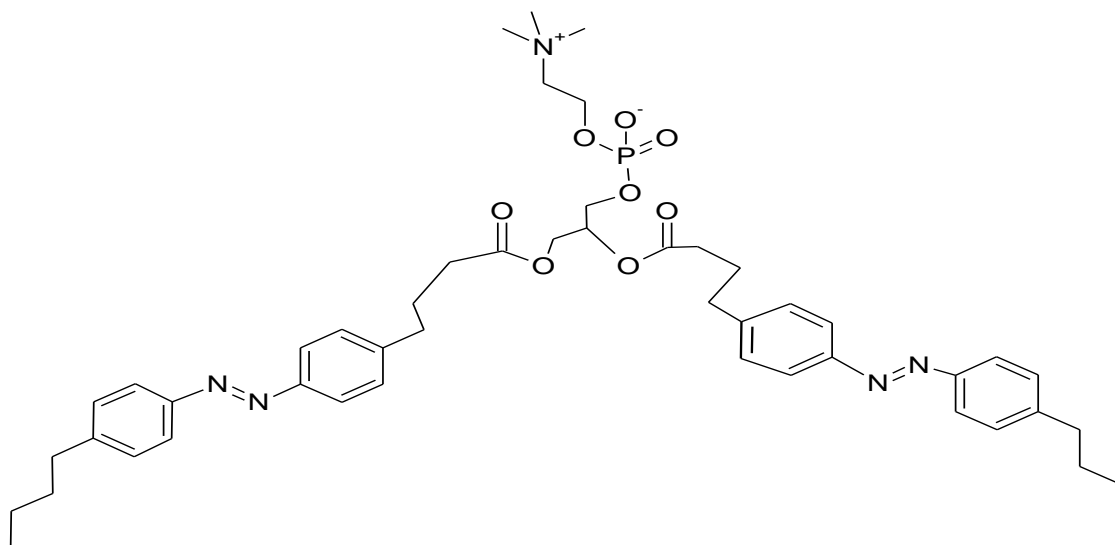
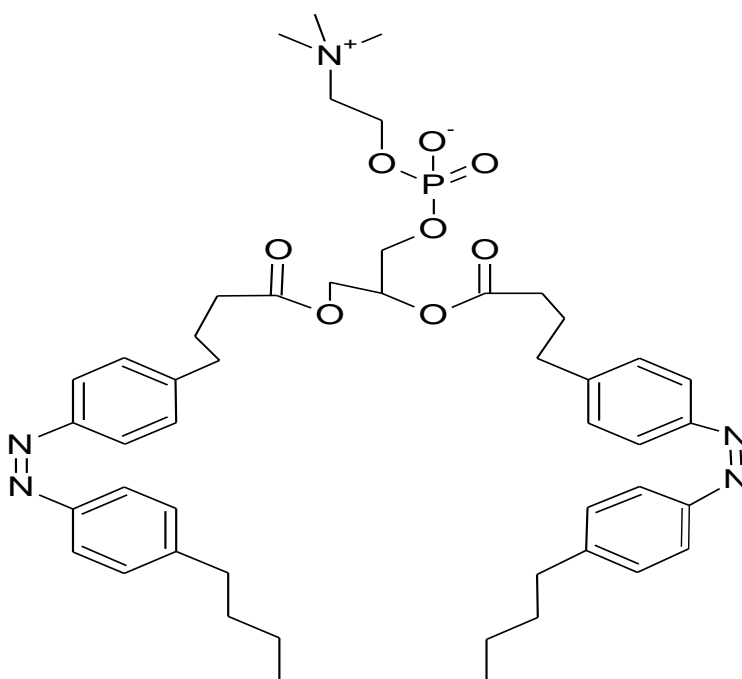
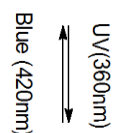


Fig 2.10: The reaction mechanism involved in the synthesis of Bis-Azo Phosphatidylcholine.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine



Trans- isomer of Bis-Azo Phosphatidylcholine



Cis- isomer of Bis-Azo Phosphatidylcholine

Fig 2.11: Stereochemistry of Bis-Azo Phosphatidylcholine.

2.4.3.1. Stereochemistry of Bis-Azo Phosphatidylcholine

The photoisomerization of Bis-Azo Phosphatidylcholine occurs due to the presence of the azo group ($-N=N-$) in conjugation with two phenyl substituents (Morgan et al., 1987b; Morgan et al., 1985; Sandhu et al., 1986). The presence of double bonds between the two nitrogen atoms gives rise to two different isomers i.e. E- or Z- configuration. Bisby and co-workers (Bisby et al., 2000a) demonstrated that in the presence of UV light (about 360 nm), the more stable trans-isomer of Bis-Azo Phosphatidylcholine is transformed into the less stable cis-isomer of Bis-Azo Phosphatidylcholine, while in presence of visible light (about 420 nm) the cis-isomer is transformed into trans-isomer (Fig 2.11). This cis-trans transformation plays a key role in case of drug delivery.

2.4.3.2. NMR spectroscopy

Due to strong overlaps of peaks in the Bis-Azo Phosphatidylcholine structure, it can be very difficult to fully interpret 1H NMR and ^{13}C NMR spectra, hence it was decided to predict the NMR spectra of Bis-Azo PC from chemical database software and compare it with the product spectra. This would confirm whether the reaction was successful or not. These results are noted in section 2.3.3.3. Along with 1H NMR and ^{13}C NMR, the resultant product was also analysed by attached proton test (APT).

The Attached Proton Test (APT) experiment is a common way to assign C-H multiplicities in ^{13}C NMR spectra. It consists of a 90° pulse that creates transverse magnetization; followed by a 180° pulse in the middle of the evolution period (serves to refocus the chemical shift). It provides the information for all sorts of carbons in one experiment (Siegmar Braun, 1998).

Depending on the number of hydrogens bound to a carbon atom, n, CH_n spin vectors evolve differently after the initial pulse. If the delay t is set to $1/J$, CH and CH_3 vectors have opposite phases compared to C and CH_2 . Therefore, the phase of CH and CH_3 peaks is 180° different from C and CH_2 peaks. It is slightly less sensitive than DEPT but a single experiment shows all carbon signals at once unlike DEPT that suppresses quaternary carbons and requires up to three different acquisitions to yield full results.

APT result of Bis-Azo Phosphatidylcholine:

Fig 2.12 shows the APT spectra of Bis-Azo Phosphatidylcholine (Refer to Fig 2.11 for structure):

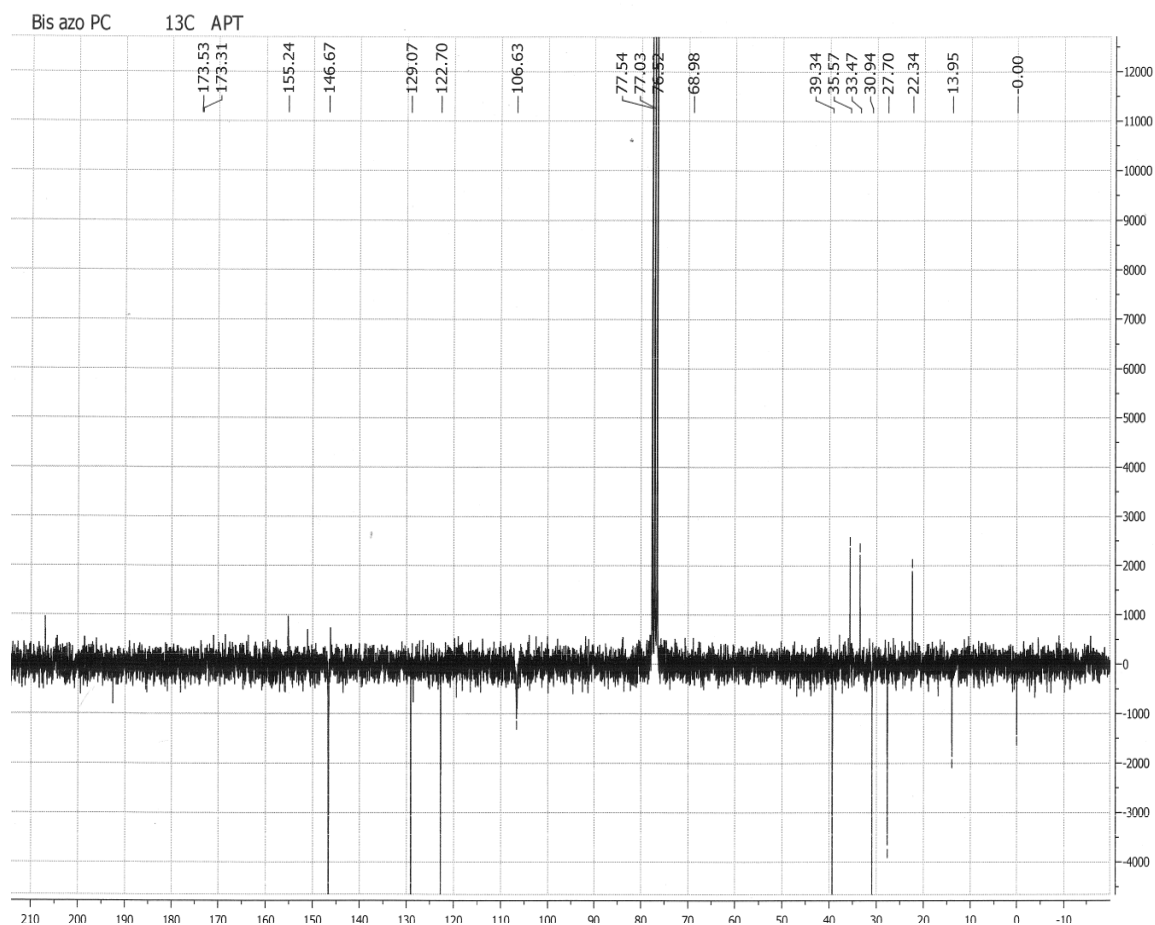


Fig 2.12: APT spectrum of Bis-Azo Phosphatidylcholine showing CH and CH₃ positive while CH₂ and quaternary C are negative.

As evident from the Fig 2.12, in an APT experiment for Bis-Azo Phosphatidylcholine (Refer to Fig 2.11), positive signals were obtained for methine (CH) and methyl (CH₃), whereas negative signals were obtained for quaternary (C) and methylene (CH₂).

2.4.3.3. UV spectroscopy

UV absorption spectra of Bis-Azo Phosphatidylcholine had maxima at 256 nm and 332 nm. The maxima was observed at 256 nm because of the $\pi - \pi^*$ transition of benzene chromophore and at 332 nm because of the $n - \pi^*$ transition between benzene and azo chromophore. From UV analysis, it is apparent that resultant product showed a shorter wavelength shift, i.e. blue shift.

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

2.4.3.4. IR spectroscopy

IR analysis of Bis-Azo Phosphatidylcholine (Bis-Azo PC) was performed on a Mattson 3000 FTIR spectrophotometer using KBr disc method. Fig 2.13 shows the IR spectrum of Bis-Azo Phosphatidylcholine (Refer to Fig 2.11 for structure).

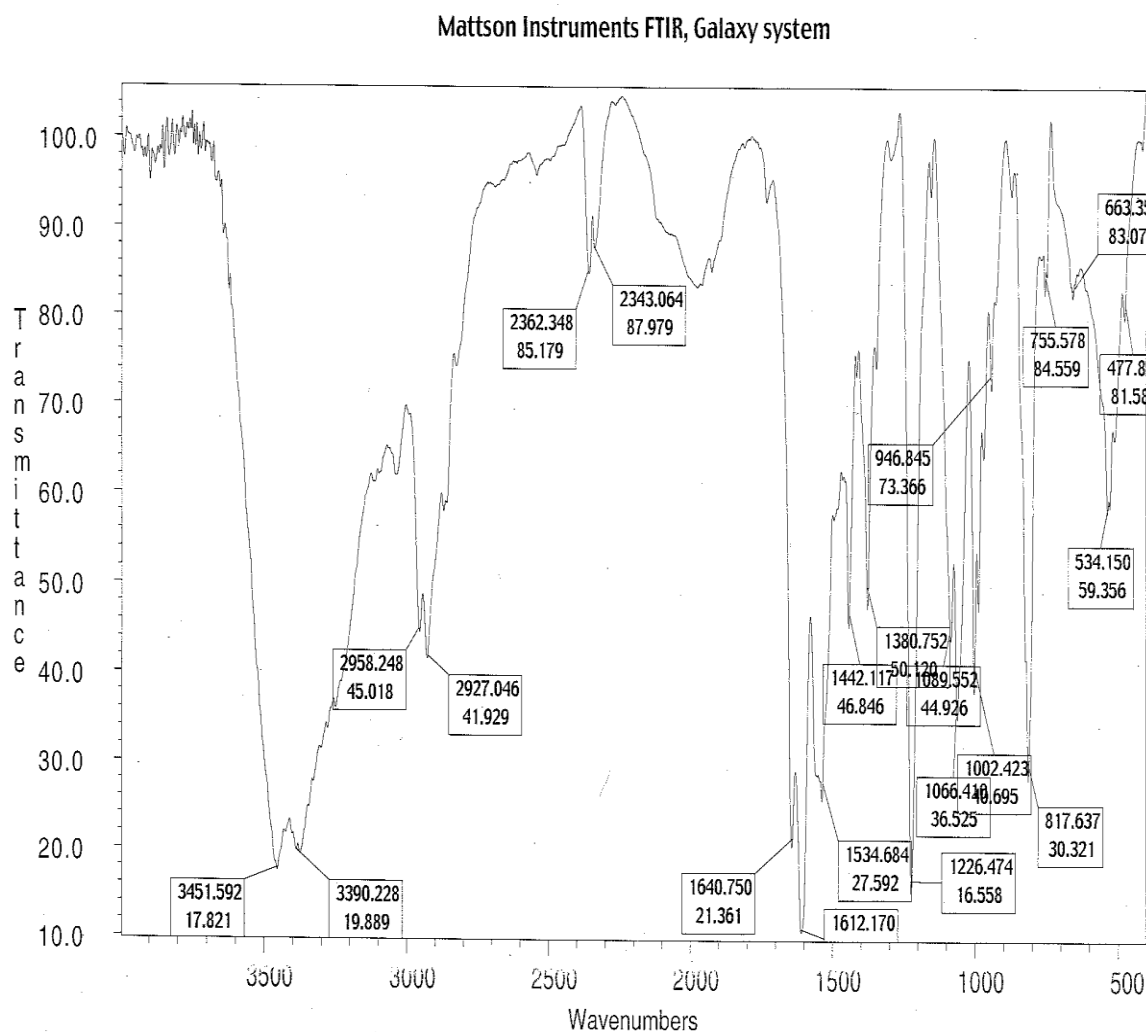


Fig 2.13: IR spectrum of Bis-Azo Phosphatidylcholine

As depicted from the Fig 2.13, the IR showed important bands at 663 cm⁻¹, 817 cm⁻¹, 946 cm⁻¹, 1066 cm⁻¹ (C-C stretching), 1226 cm⁻¹, 1380 cm⁻¹ (C-N stretching), 1612 cm⁻¹, 1534 cm⁻¹ (Aromatic rings), 1640 cm⁻¹ (C=O stretching), 2362 cm⁻¹, 2343 cm⁻¹ (C=C stretching), 2927 cm⁻¹ (C-H stretching), 2958 cm⁻¹ (CH₃) and 3390 cm⁻¹ (O-H stretching).

Chapter 2-Synthesis of Bis-Azo Phosphatidylcholine

2.4.3.5. Mass spectrometry

With the intention to determine the exact mass of Bis-Azo Phosphatidylcholine, the WATERS LCT premier ESI-TOF-MS instrument was used. It determines the masses of compounds by analysing the mass-to-charge ratio of the charged species obtained from the compound after electrospray-ionization (Fenn et al., 1989). The Bis-Azo PC sample was dissolved in methanol prior to mass spectroscopy analysis. The sample was loaded onto the instrument and the molecular components in solution were vaporized into the gas phase by bombarding them with beam of electrons which produce an ionic molecule or ionic fragments of the original species. These ionic molecules were then passed through the magnetic or electric fields to the mass detector, where they were isolated as per their mass to charge ratio (m/e) and thus easily detected (Tanaka et al., 1988). The spectrum (Fig. 2.14) below clearly demonstrates the ion signals which were generated from Bis-Azo Phosphatidylcholine.

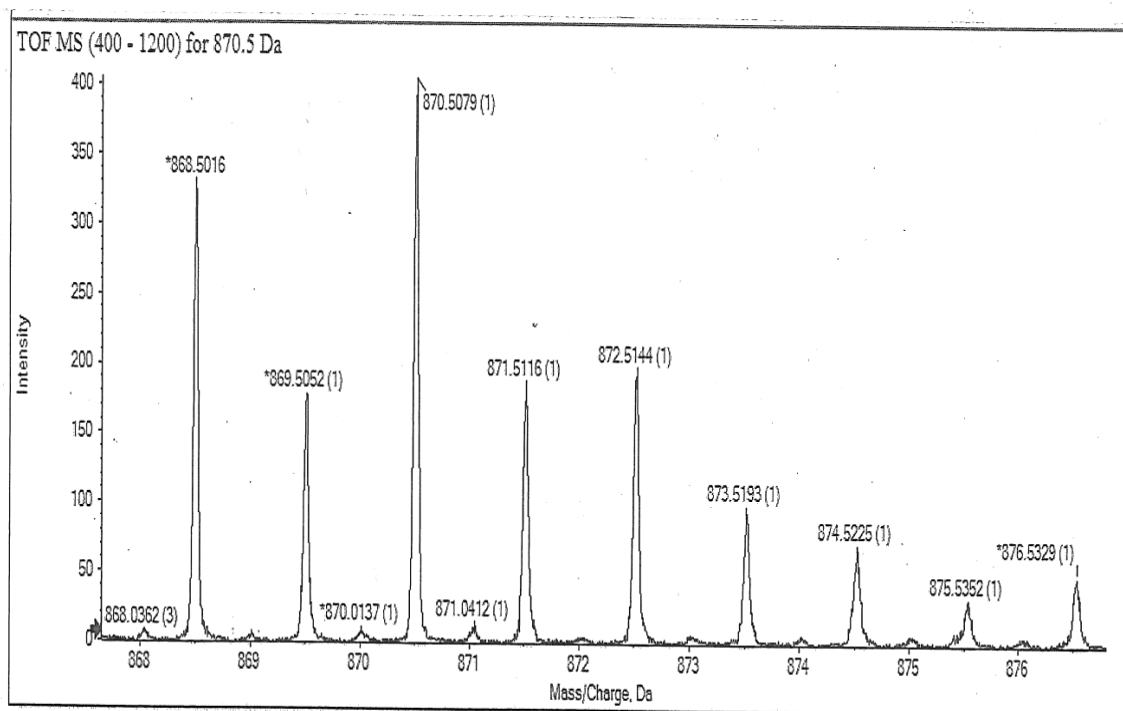


Fig 2.14: Mass spectrum of Bis-Azo Phosphatidylcholine.

Molecular formula of Bis-Azo Phosphatidylcholine is $[C_{48}H_{64}N_5O_8P]$ with a calculated monoisotopic mass of 870.02. As depicted from Fig 2.15, the obtained mass of the product was 870.50.

It is evident from the NMR,UV,IR and mass spectrometry results that Bis-Azo Phosphatidylcholine was successfully synthesized and characterized by the above methods (Fig 2.3, see section - 2).

2.5. Conclusion

In Conclusion, Bis-Azo Phosphatidylcholine was successfully prepared. This synthetic approach could advance the synthesis of azo based photosensitive liposome. There were a lot of difficulties encountered in Bis-Azo Phosphatidylcholine synthesis. Despite of these difficulties, Bis-Azo Phosphatidylcholine was synthesized successfully.

Proton and carbon NMR results showed all desired peaks that can confirm the structure of Bis-Azo Phosphatidylcholine. Moreover, NMR spectroscopy can be used to differentiate trans and cis isomers, and this was confirmed by the coupling constant of trans isomer was larger than the coupling constant of cis isomer. UV and IR analysis verified the presence of N=N chromophore and functional groups such as OH, C=C, C-H, CH₃, aromatic ring etc. These results also contribute to the presence of Bis-Azo Phosphatidylcholine molecule.

Mass spectrometry proved the correct mass of the synthesised Bis-Azo Phosphatidylcholine. That means the route for the synthesis of Bis-Azo Phosphatidylcholine was workable. The developed synthetic strategy in this project could also be applied to prepare other new photosensitive lipid derivatives.

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CHAPTER 3

Development of Photosensitive Liposomes for the Controlled Release of Bovine Serum Albumin-Fluorescein Isothiocyanate (BSA-FITC)

Chapter 3-Development of Photosensitive Liposomes for the Controlled Release of Bovine Serum Albumin-Fluorescein Isothiocyanate (BSA-FITC)

3.1 Introduction

Liposomes are established as drug delivery systems with products such as DaunoXome[®], Ambisome[®], Visudyne[®], Thermodox[®]. Some of them have been used as potential carriers for controlled release of drugs (Torchilin, 2005). In the past, scientists have reported that the controlled release of drugs from liposomes can be triggered by a variety of external stimuli such as heat (Gaber et al., 1996), light (Pidgeon and Hunt, 1983) and pH (Yatvin et al., 1980). Later on, attention has been directed towards the development of photosensitive liposomes which release their contents in response to irradiation with an appropriate wavelength of light. Photosensitive liposomes can be formulated by two methods: incorporation of photosensitive lipid into the bilayer membranes, for instance, incorporation of azobenzene based moieties (Lei and Hurst, 1999) and using nanoparticles to channel light energy into heat energy or mechanical energy, such as, gold nanoparticles (Park et al., 2006).

This chapter primarily focuses on the formulation of photosensitive liposomes by incorporation of photosensitive lipid into the bilayer membranes. As discussed earlier (Section 1.3.3), photosensitive lipid is composed of three parts: a polar hydrophilic head group, a chromophoric group, which is responsible for light absorption and photoisomerization, and lastly, a hydrophobic tail. Upon UV irradiation, photosensitive liposomes incorporating photosensitive lipids become unstable due to photoisomerisation of the chromophore. This process results in the disruption of the lamellar integrity and the fast release of liposome-entrapped drugs (Fig 3.1) (Shum et al., 2001). Overall, the mechanism of the photo- triggered release mostly relies on the principle of membrane destabilization in response to light for the rapid release of trapped pharmaceuticals.(Wan et al., 2002; Jeong et al., 2002; Anderson and Thompson, 1992)

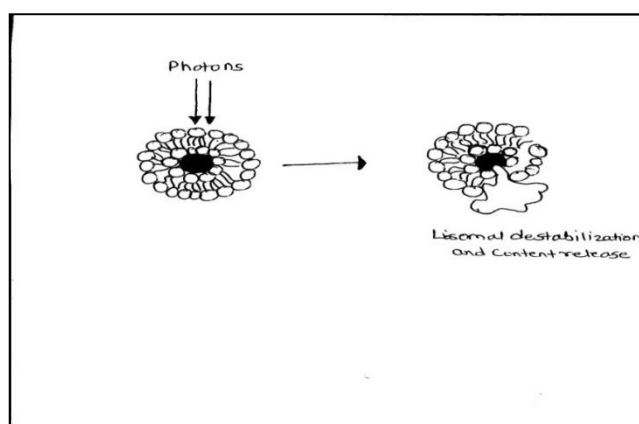


Fig 3.1: Exposure to long wavelength UV light causes rapid release of entrapped drug.

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Numerous studies (Sandhu et al., 1986; Morgan et al., 1987a) have shown that stable light-sensitive liposomes of gel phase lipids can be successfully prepared by incorporating photosensitive lipid “Bis-Azo PC”, which is a phosphatidylcholine (PC) molecule having acyl chains incorporating azobenzene moieties. However, this photosensitive lipid exists in trans-isomer under visible light illumination, and it is transformed into the cis-isomer on exposure to UV light at 366 nm (Fig 3.2) (Morgan et al., 1985). This trans-cis isomerisation is responsible for the release of liposomal contents. During this transformation, azobenzene chromophores are very compact, relatively linear molecules of low polarity, whereas in cis-form, azobenzene chromophores are non-linear with a large molecular dipole moment (Morgan et al, 1995).

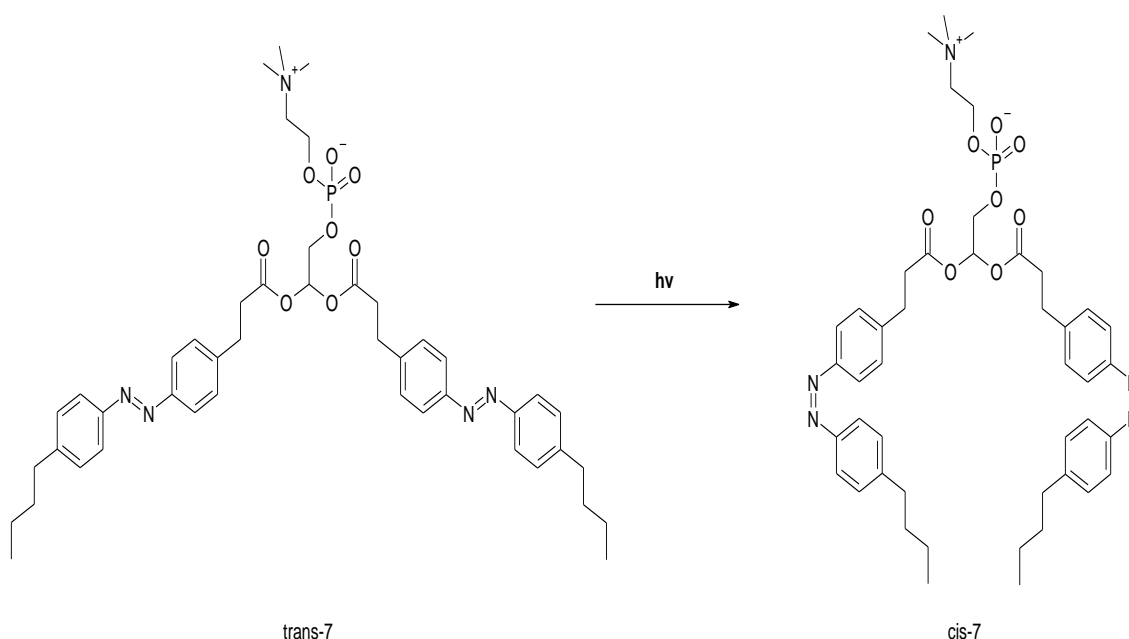


Fig 3.2: On exposure to UV light, trans-form of Bis-Azo PC gets transformed into cis- form of Bis-Azo PC [adapted from (Morgan et al., 1985)].

Liposomes containing Bis-Azo PC have been used to ‘cage’ water-soluble solutes, which include biologically important cations, such as copper and calcium (Bisby et al., 1999b). In addition, liposomes with Bis-Azo PC have been successfully used to ‘cage’ water-insoluble drugs, such as methotrexate (New 1990). Photosensitive liposomes have an advantage as they could be applied as carriers for introducing biologically active material into cells by endocytosis, thus minimising materials exposure to degradative enzymes prior to release upon trigger. Indeed, Morgan and co-workers (Morgan et al., 1987a) prepared azo based photosensitive liposomes from dipalmitoyl phosphatidylcholine (DPPC) containing 8 % (mole: mole) Bis-Azo PC, entrapping a fluorescent marker dye over a period of month with negligible release. Furthermore, they have used long

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wavelength UV to release a similar dye, within minutes, from photosensitive liposomes. Morgan and co-workers also noted that in terms of size, Bis-Azo PC resembles DPPC and that both lipids contain a highly cooperative phase transition at 41 °C.

DPPC are good building blocks for the production of liposomes due to their zwitterionic effect. In addition, phospholipids with a longer chain length possess higher encapsulation efficiency and stability than phospholipids with shorter chain length (Mohammed et al., 2004b). Hence DPPC is an ideal choice as compare to other PC. Moreover, the addition of cholesterol to this photosensitive liposomes increases stability and reduces permeability (Bisby et al., 1999b). However, cholesterol amount is vital; at small amounts, cholesterol could easily dissolve within the lipid bilayer while at large amounts (~ 50 % mol/mol), crystals of cholesterol monohydrate could be produced that lead to liposomal instability (Epand and Epand, 2003; Epand et al., 2003). Based on these results, Smith and co-workers (Smith et al., 2007) developed photosensitive interdigitation fusion vesicles (IFV) which they used for tissue engineering. In brief, they formulated IFVs from DPPC, Cholesterol and Bis-Azo PC by using interdigitation fusion vesicles method (Ahl et al., 1994) entrapping calcium ions within. Calcium ions released from this system by using light as a trigger mechanism are beneficial for the gelation of alginate i.e. tissue engineering. IFV are large unilamellar vesicles possessing diameters of 1-6 µm and entrap volumes of 20 µl/ µmoles lipid or above (Ahl et al., 1992). IFV are ideal vehicles for drug delivery as these vesicles contain the large internal volume which not only increases drug entrapment efficiency, but also increases entrapment of compounds such as radiocontrast agents (Ahl et al., 1994).

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3.2. Aim and Objectives:

Following on Smith's (Smith et al., 2007) and Ahl's work (Ahl et al., 1994), the aim of this study was to develop a novel liposomal formulation encompassing the use of photosensitive trigger release mechanism. To achieve this aim, the following objectives were accomplished:

- Incorporation of photosensitive lipid i.e. Bis-Azo PC (prepared in Chapter 2) into various liposomal formulations (DPPC:Chol) and entrapment of a protein marker i.e. Bovine serum albumin conjugated with fluorescent marker (BSA-FITC). Bovine serum albumin is a protein which is obtained from cows and generally used as a protein concentration standard in lab experiments (Deb, 2001; Hawkins and Dugaiczyk, 1982). Therefore, BSA has been used in various biochemical applications including microbial culture (Francis, 2010), ELISAs (Enzyme-Linked Immunosorbent Assay) (Xiao and Isaacs, 2012) and immunohistochemistry (Habeeb, 1978).
- Use various UV lights leading to the selection of the ideal liposomal formulation.
- Determine liposomal stability in order to detect the leakage of the protein marker.

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3.3. Materials and Methods

3.3.1. Materials

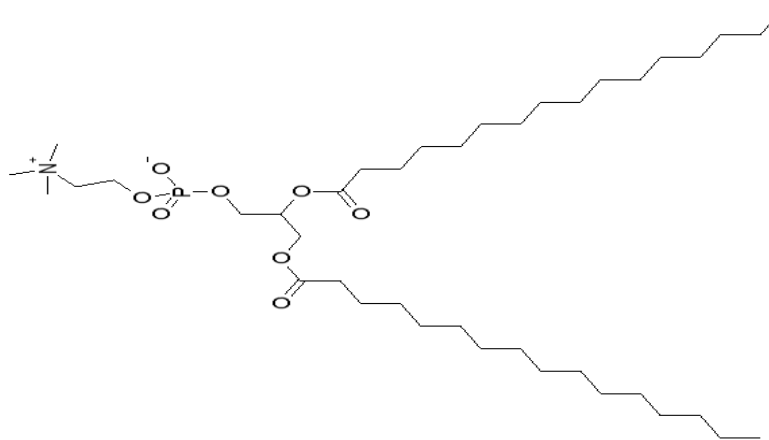
3.3.1.1. Chemicals

Chloroform and methanol were purchased from Fisher Scientific, Loughborough, UK. Bovine serum albumin-fluorescein isothiocyanate, Triton- X and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich, Poole, Dorset, UK. Distilled water was used throughout. All the chemicals used were of analytical grade.

3.3.1.2. Lipids

1, 2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DPPC), with a chemical structure shown in Fig.3.3 (a) and cholesterol, with a chemical structure shown in Fig 3.3 (b), were purchased from Avanti Polar lipids, Alabama, USA.

(a)



(b)

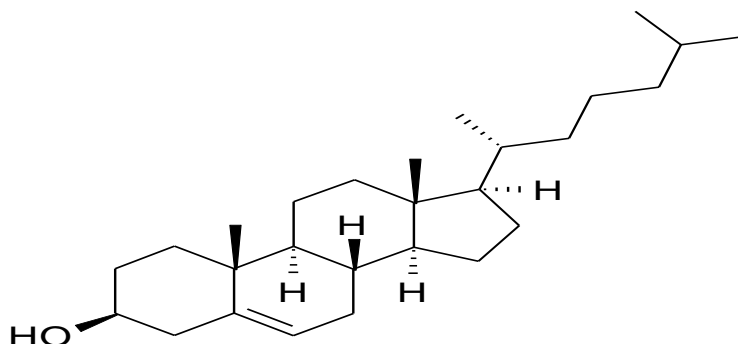


Fig 3.3: Chemical structures of (a) DPPC; (b) cholesterol.

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3.3.2. Methods

All tests were conducted at room (21-24 °C) except where particular conditions were required.

3.3.2.1. Synthesis of photosensitive lipid Bis- Azo PC

The chemical synthesis of photosensitive lipid 1, 2-bis (4-(*n*-butyl) phenylazo-4'-phenylbutyroyl) phosphatidylcholine (Bis-Azo PC) was that of Sandhu (Sandhu et al., 1986) as described in Chapter 2 (Section 2.3.3.4).

3.3.2.2. Preparation of multilamellar vesicles (MLV)

MLVs were generated using thin film hydration method (Fig 3.4) (Bangham et al., 1965). Lipids such as DPPC, cholesterol and photosensitive lipid Bis-Azo PC were dissolved in a 9:1 (v/v) mixture of chloroform and methanol at a concentration of 10 mg/mL. As these lipids are sparingly soluble in either solvent, a mixture of chloroform and methanol was used to obtain complete dissolution of the lipid components. It should be noted all liposomes were prepared in PBS because the ionic strength and the pH (7.4) of PBS are almost identical with conditions met inside the human body.



Fig 3.4: MLV formation by thin film hydration method [taken from (Bangham et al., 1965)].

Liposomes containing controlled amount of DPPC (16 μ moles), with or without Bis-Azo PC (1 μ moles) and varying amount of cholesterol (0-5 μ moles) were formulated.

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The lipid compositions of the different formulations utilised in this study are divided into 6 groups and listed in Table 3.1.

Table 3.1 IFV formulations based on the liposome composition

Group	DPPC (μmoles)	Cholesterol (μmoles)	Bis-Azo PC (μmoles)
1	16	0	0
2	16	0	1
3	16	2	0
4	16	2	1
5	16	4	1
6	16	5	1

Briefly, the dissolved lipids depending on the liposomal contents (Table 3.1) were added into a 50 mL round bottom flask and the solvents were evaporated on a rotary evaporator (Buchirota vapor-R) in order to form a thin dry lipid film which was further dried with nitrogen to remove any residual solvent. Thereafter, the dry film was hydrated with 2 mL of a 10 μg/mL hydration media (PBS) and then mixed, at intermittent vortexing, with Bovine serum albumin-Fluorescein Isothiocyanate which had been prewarmed above the transition temperature [T_m (41 ± 1 °C)] of the lipids, until a milky coloured suspension was obtained. The suspension was allowed to stand for about 30 min in a water bath above T_m with intermittent periods of vortex, during which multilamellar vesicles were formed. Whole MLV formation was conducted above the transition temperature (T_m) of the lipids with the intention that a lipid is in the liquid crystalline phase for MLV formation.

3.3.2.3. Preparation of small unilamellar vesicles (SUV)

Small unilamellar vesicles were prepared from the MLV suspension prepared as described in section (3.3.2.2) with further sonication using probe and bath sonicator.

For Probe sonication, the MLV suspension was sonicated in Soniprep 150 (MSE, UK) at $>T_m$ using a titanium probe (3 mm diameter) which was slightly immersed into the suspension. With the high energy input, the milky suspension of MLV was converted to optically clear suspension of SUV. Based on the liposome content, the liposomal samples were sonicated for 30 s, 1 min and 2 min at a power rating of 6 units. After sonication, the SUV was left to stand at the corresponding T_m for 15 min.

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In case of bath sonication, MLV suspension was placed in bath sonicator (Fisher scientific, UK) and then sonicated at 55 °C until optically clear. Again based on the liposome content, the liposomal formulations were bath sonicated at 55 °C for 25 min, 35 min, 45 min and 1 h respectively. After sonication, the SUV was left to stand at the corresponding T_m for 15 min.

3.3.2.4. Preparation of interdigitation fusion vesicles (IFV)

Interdigitation fusion vesicles were prepared by an interdigitation fusion method as described by Ahl and co-workers (Ahl et al., 1994). Briefly, absolute ethanol was added to a SUV suspension (prepared as describe in section 3.3.2.3) while stirring at room temperature until the ethanol concentration in the sample was 4M. The samples were instantly vortexed. This process quickly altered the transparent SUV suspension into an extremely viscous, opaque white suspension of interdigitated phospholipid sheets. Following the inclusion of absolute ethanol, the samples were sealed and incubated for 15 min at room temperature, then incubated for 15 min at 50 °C ($> T_m$ of the lipid). The caps of the sample vials were then loosened to allow ethanol evaporation, and the incubations were continued for another 30 min at the same temperature. After this time, the samples were bubbled at 50 °C ($> T_m$ of the lipid) with a gentle stream of N_2 to remove ethanol. Subsequently, External protein was then removed from the IFV suspension by repeated centrifugation (three times), decanting the supernatant and washing the pellet with isosmotic buffered saline until no protein could be detected in the supernatant. Lastly, resultant pellet were suspended with PBS and the vesicles were stored at 4 °C.

3.3.2.5. Liposome characterization

3.3.2.5.1. Determination of particle size

The size distribution of liposomes play a key role when liposomes are applied as drug delivery systems (Franzen and Ostergaard, 2012). In this investigation, the sizes of MLV and IFV were characterised using the Sympatec (Helos, UK) particle sizer. In brief, for the size measurement, the liposomal suspension was diluted up to 100 times. Following this, 100 μ L of a liposome formulation was pipetted into a glass quartz cuvette (with a marked signal of at least 15 % being mandatory for an ample reading) and stirred at 1200 rpm to reduce the inter particulate aggregation, and thereafter, the laser beam was focused using Helos software, and the experiment was repeated in triplicate (Plessis et al., 1994). The particle size and polydispersity index (PI) measurements were measured in triplicate. Sympatec (Helos, UK) uses a Helium Neon laser of 632.8 nm (Sympatec) with an R2 lens for laser generation and employs the Phillips-Twoney method (Sympatec Limited, UK) for calculation of particle size distribution.

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Due to the very small size of SUV compared to MLV and IFV, the z-average diameter of SUV were measured using a Zetaplus (Brookhaven Instruments, UK) instead of Sympatec. During the measurement, 100 μ L of SUV suspension was diluted to 4.5 mL using double-distilled water and the z-average diameter of SUV were recorded at 25 $^{\circ}$ C. Measurements were reported as a mean value of three readings and each reading was an average value of measurements recorded for 3 min.

3.3.2.5.2. Determination of zeta potential

Zeta potential of a liposome is the overall charge that liposome acquires in a particular medium. In short, the process of an indirect measurement of the surface charge of a liposome is termed as zeta potential (Perrie et al., 2004). Knowledge of the zeta potential of vesicles can be beneficial in determining the fate of the vesicles *in vivo* (Jones, 1995). In this study, the electrophoretic mobility and zeta potential of liposomal dispersions were analysed by photon correlation spectroscopy using Zetaplus (Brookhaven Instruments, UK) in 0.001M PBS (pH=7.4) at 25 $^{\circ}$ C. 100 μ L of liposomal dispersions were diluted to 4.5 ml using 0.001M PBS prior to analysis. The final measurements were the mean values of ten readings.

3.3.2.6. Removal of un-entrapped protein marker through centrifugation

In bid to remove un-entrapped (BSA-FITC), the liposomal formulations (2 mL) were placed into 3.9 mL of Beckman coulter centrifuge tubes, which were heat sealed and placed into Beckman coulter ultracentrifuge equipped with TLN-100 rotor. The centrifuge cycle began once the vacuum was switched on and had reached < 50. Figure 3.5 illustrates the step to remove un-entrapped protein marker through centrifugation. The ultra-centrifuge was used for speeds of 27, 000 g with the TLN-100 rotor for 3.9 mL tubes.

Protocol 1	
1) Place 2 mL of liposomal samples in 3.9 mL of Beckman centrifuge tubes and make up the remaining volume with PBS.	× 3
2) Then Centrifuge at 27,000 g for 30 min at 4 $^{\circ}$ C.	
3) Remove supernatant and resuspend pellet in 2 mL of PBS.	

Fig 3.5: Protocols used for the removal of un-entrapped protein through ultracentrifugation. Protocol 1 was repeated for three times in order to remove minor amount of un-entrapped BSA-FITC.

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3.3.2.7. Determination of protein entrapment efficiency in IFV

The loading efficiency of Bovine serum albumin conjugated with fluorescence isothiocyanate (BSA-FITC) was determined by measuring the non-incorporated present in the hydration medium and wash media after separation of liposomes by centrifugation at 27,000 g for 30 min. Briefly, samples were prepared by addition of 500 µL aliquot of liposome suspension to 1 ml solution of phosphate buffer saline followed by addition of 500 µL Triton X-100 (0.1 % solution). Triton X-100 solubilized the liposome membranes and induced release of entrapped contents. All samples were diluted, centrifuged and the supernatant was measured. The fluorescent protein content in the supernatant was determined by fluorescence spectroscopy (Perkin-Elmer Life Sciences) at excitation and emission wavelengths of 490 and 515 nm, respectively. The wavelength scan and optimisation of calibration curve of BSA-FITC through fluorescence spectroscopy are demonstrated in Appendix 1.

The protein entrapment efficiency (% EE) was calculated using the following formula –

$$\% \text{ EE} = \left(1 - \frac{F}{F_t}\right) \times 100 \dots\dots\dots \text{Equation (3.1)}$$

where F is the fluorescence prior to the addition of 0.1 % Triton X-100 and F_t is the fluorescence after the addition of Triton X-100

3.3.2.8. Photo-triggered release study of fluorescent protein (BSA-FITC):

The liposome samples was irradiated using three different lamps, a UV curing Flood Lamp, a ROHS 36 w professional UV curing lamp and a Nichia NSHU590E UV LED lamp (Fig 3.6). The emission characteristics of all these lamps were quantified using Accucal 50 UV intensity meter resulting in a calculated intensity (I) of 1.81 x 20 mW/cm², 2.94 x 20 mW/cm² and 0.74 x 20 mW/cm² respectively. In order to study the controlled release of fluorescent protein (BSA-FITC) from IFV, liposomes samples, stored in the dark prior to the study, were irradiated for different time intervals i.e. 0, 1 and 3 min before being evaluated using the fluorescent assay described above (section 3.3.2.7).

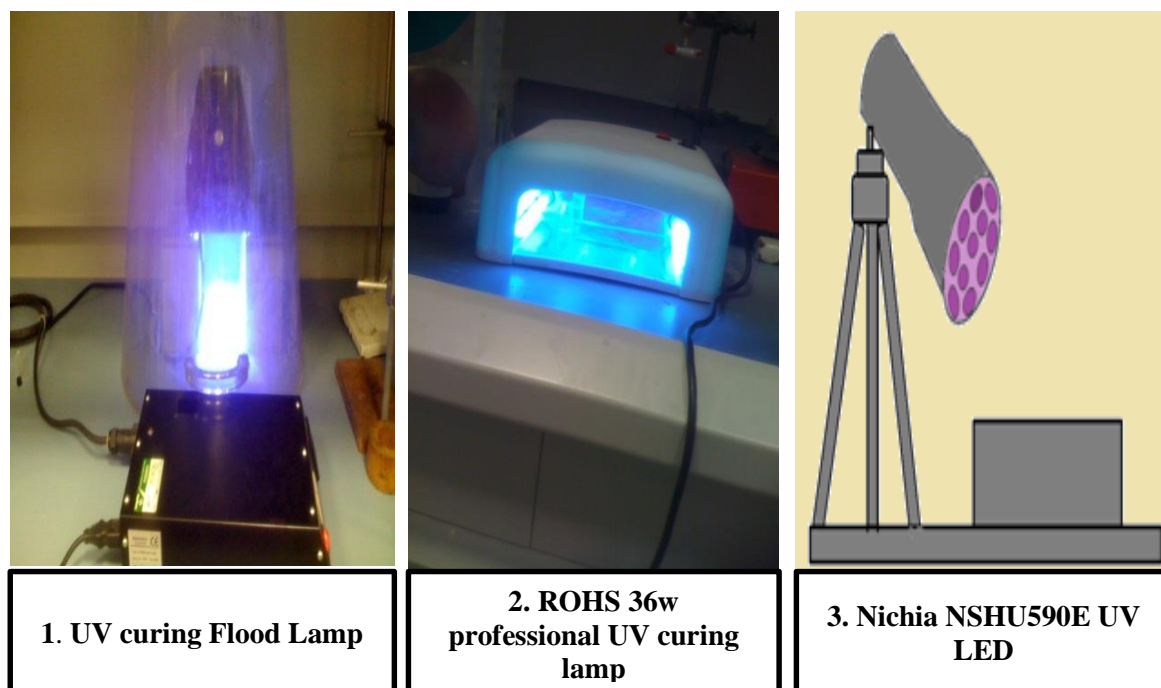


Fig 3.6: Exposure of UV light from three different lamp, lamp 1- UV curing Flood Lamp; lamp 2- ROHS 36w professional UV curing lamp and lamp 3- Nichia NSHU590E UV LED.

3.3.2.9. Determination of 100 % release of fluorescent protein (BSA-FITC) from IFV using ROHS 36 w professional UV curing lamp.

To measure protein (BSA-FITC) release from IFV, liposomes samples that had been stored in the dark were taken and irradiated using ROHS 36 w professional UV curing lamp for different time intervals i.e. 0, 1, 3, 6, 9, 12 and 15 min. The samples were diluted, centrifuged and the supernatant used to determine the fluorescent protein content using fluorescent assay as described above.

3.3.2.10. Determination of IFV short term stability

IFV stability is an important aspect that is considered during the development of photosensitive liposomes. It is well established that photosensitive liposomes containing Bis-Azo PC are not stable when stored in the dark at room temperature (Smith et al., 2007). Therefore, in order to evaluate the short term stability of the formulated IFV during storage, IFV were effectively stored at room temperature ($\sim 23^{\circ}\text{C}$) for up to 15 min, 24 h and 48 h. Fluorescent Protein (BSA-FITC) leakage was checked by fluorescence spectroscopy. In brief, after 15 min, 24 and 48 h, all samples were diluted, centrifuged and the supernatant was analysed for fluorescent protein content using fluorescence assay described above.

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3.3.2.11. Determination of IFV long term stability

With the intention to estimate the long term stability, the formulated IFV were stored at fridge temperature (6 °C) in the dark for one and two weeks. Fluorescent Protein (BSA-FITC) leakage was checked by fluorescence spectroscopy. Analysis was carried out after one week and two weeks of sample storage. The samples were diluted, centrifuged and the supernatant used for fluorescent protein content determination using fluorescence assay described above.

3.3.2.12. Confocal Microscopy

A Leica confocal microscope was used and MLV, SUV, IFV with or without BSA-FITC were examined by a 60 X oil objective along with Helium and Argon lasers. Following the formulation step, the un-entrapped BSA-FITC was successfully removed through centrifugation and subsequently liposomal formulation was placed into a microslide and immediate drying out of sample was avoided by mounting cover slip on it. Furthermore, Fluorescein isothiocyanate (FITC) filters were used for the BSA-FITC analysis.

3.3.2.13. Statistical analysis

Graph-Pad Instant software (v.6.02, GraphPad Software, San Diego, CA) was used for statistical analysis. Particularly, using one-way analysis of variance (ANOVA) and a multiple comparison post-test Tukey for determining statistical significance (set at $P < 0.05$) between data sets.

3.4. Results and Discussion

Data obtained in previous studies (Fig 3.7) show that IFV can be easily prepared by interdigitation fusion method (Ahl et al., 1994). This technique requires organic solvent i.e. ethanol which induces interdigitation –fusion of SUV into large interdigitated lipid sheets, which are subsequently annealed into vesicles. Based on this methodology, interdigitation fusion vesicles (IFV) were generated following three steps:

- 1) Multilamellar vesicles (MLV) were prepared using a method based on dry film hydration (Bangham et al., 1965).
- 2) Small unilamellar vesicles (SUV) were formulated through probe and bath sonications.
- 3) Interdigitation fusion vesicles were prepared using Interdigitation fusion method (Ahl et al., 1994).



Fig 3.7: Formation of IFV from SUV [taken from (Ahl et al., 1994)].

As discussed previously (Sections 1.2.2 and 1.2.3.), MLV are more stable than SUV and are easily converted into SUV through sonication. Therefore, in order to study the effect of sonication on SUV formation, this study used both types of sonication. Probe sonication was done using Soniprep 150 (MSE, UK) while bath sonication was done using ultrasonic bath (Fisher scientific, UK).

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3.4.1. Effect of sonication on SUV formation

SUV were obtained through sonication, using both probe and bath sonication methods, and were used to investigate the effect of cholesterol level on SUV formation. It is well known that probe tip sonicator delivers high energy input to the lipid suspension which instantly converts the MLV suspension into SUV (Papahadjopoulos and Watkins, 1967). However, probe sonication has limitations, such as, SUV dispersions contamination with metal ions such as titanium particles, limited control of MLV temperature of due to open system as probe is immersed into the MLV dispersion (Barenholz and Lichtenberg, 2009). However, SUV that are produced by bath sonication are usually metastable (Lasch et al., 2003). The finding in terms of sonication time is shown in Table 3.2 and in terms of resultant liposomes size, zeta potential are shown in Fig 3.8.

Table 3.2: Formation of SUV through sonication

Formulation	Probe sonication time	Bath sonication time at 55°C
DPPC (16 μ moles)	0.5 min	25 min
DPPC:Bis-Azo PC (16 μ moles:1 μ moles)	0.5 min	25 min
DPPC:Cholesterol (16 μ moles:2 μ moles)	1 min	35 min
DPPC:Cholesterol:Bis-Azo PC (16 μ moles:2 μ moles:1 μ moles)	1 min	35 min
DPPC:Cholesterol:Bis-Azo PC (16 μ moles:4 μ moles:1 μ moles)	1.5 min	45 min
DPPC:Cholesterol:Bis-Azo PC (16 μ moles:5 μ moles:1 μ moles)	2 min	60 min

Table 3.2 illustrates that SUV formation through probe sonication and bath sonication using 16 μ moles of DPPC, with or without 1 μ moles of Bis-Azo PC and varying cholesterol content (0-5 μ moles). Total volume was 2 mL for each formulation.

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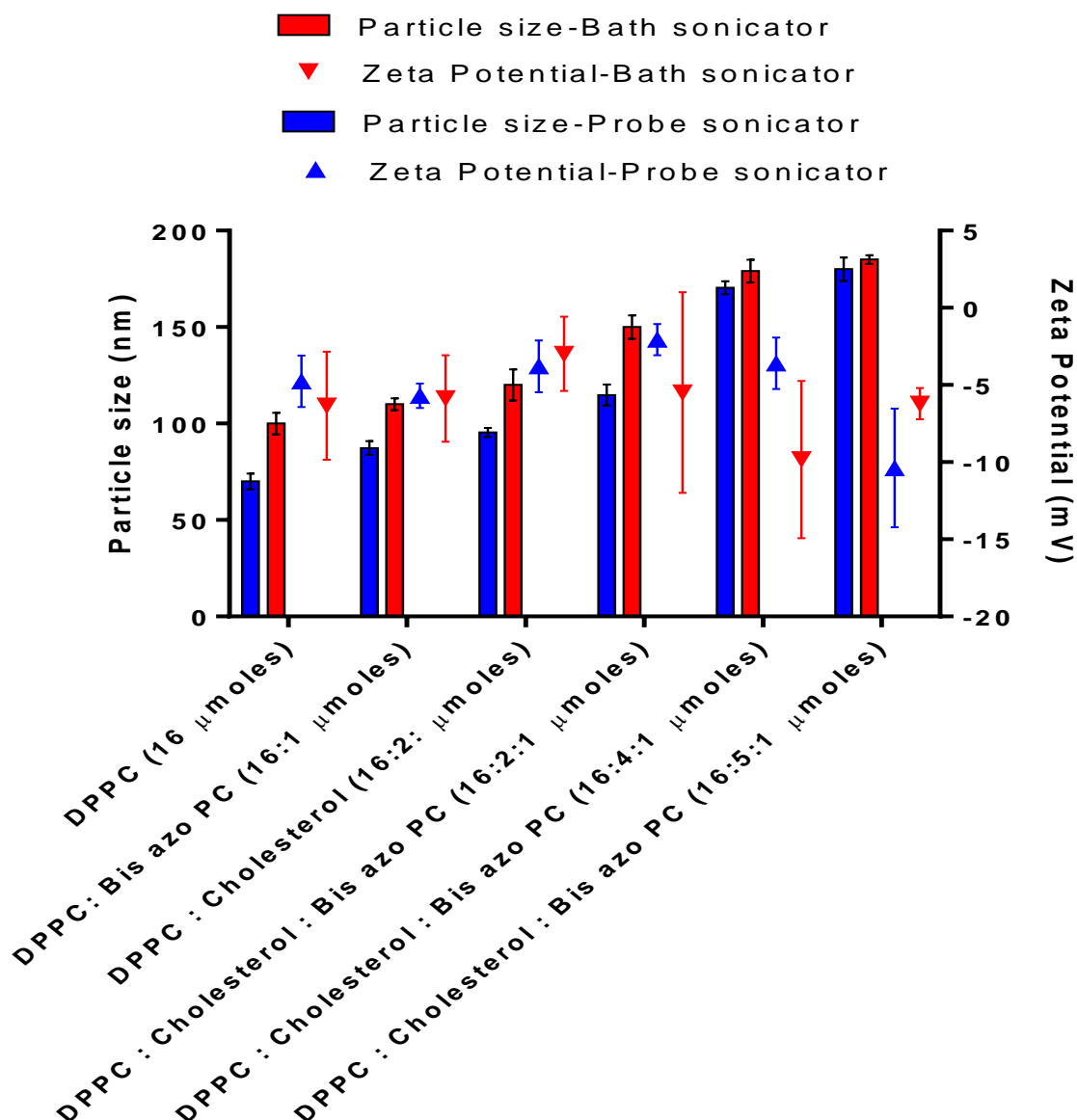


Fig 3.8: Particle size (nm) and zeta potential (mV) of various small unilamellar vesicle formulations prepared via bath and probe sonication.

SUV formulations were prepared by using bath sonicator and probe sonicator. Particle size analysis and zeta potential measurement was measured at 25 °C using a Brookhaven Zeta Plus. The addition of cholesterol into the formulations showed substantial interference of the average size of the vesicles. The data are expressed as means \pm standard deviation of three independent experiments. Significance was measured by one-way ANOVA (** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$) and represent a significant increment in vesicle size due to the substitution of cholesterol into the formulations.

As can be seen from Table 3.2, the time for SUV formation varies with the amount of cholesterol in the formulations. Indeed, it is also evident from the results that increasing amount of cholesterol increases sonication time and SUV can be easily obtained from 16 μ moles of DPPC and 16 μ moles

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of DPPC: 1 μ moles of Bis-Azo PC compared to other formulations. Moreover, the sonication times for SUV produced from 16 μ moles of DPPC and 16 μ moles of DPPC: 1 μ moles of Bis-Azo PC are almost identical. Therefore, inclusion of photosensitive lipid Bis-Azo PC has no effect on sonication time. In contrast, sonication time for SUV obtained from 16 μ moles of DPPC: 2 μ moles of cholesterol; 16 μ moles of DPPC: 2 μ moles of cholesterol: 1 μ moles of Bis-Azo PC; 16 μ moles of DPPC: 4 μ moles of cholesterol: 1 μ moles of Bis-Azo PC and 16 μ moles of DPPC: 5 μ moles of cholesterol: 1 μ moles of Bis-Azo PC varies as the cholesterol amount increases. The addition of cholesterol not only gives rigidity to the bilayer through lipid rearrangement process, but also improves the stability of liposomes. This is supported by previous experiments (Demel and De Kruffy, 1976; Senior, 1987; Kirby et al., 1980) in which they proved that the presence of cholesterol in liposome membranes resulted in increased packing densities of phospholipids.

Data in Table 3.2 also suggest that SUV can be easily obtained from probe sonication within shorter periods of time when compared to bath sonication. Despite the fact that the processing time for SUV formation through bath sonication is more, this method avoids the contamination with titanium particles, which are produced in probe sonication.

As depicted from Fig 3.8, particle size for SUV exhibited from 16 μ moles of DPPC and 16 μ moles of DPPC: 1 μ moles of Bis-Azo PC are similar whereas the particle size for SUV revealed from 16 μ moles of DPPC: 2 μ moles of cholesterol; 16 μ moles of DPPC: 2 μ moles of cholesterol: 1 μ moles of Bis-Azo PC; 16 μ moles of DPPC: 4 μ moles of cholesterol: 1 μ moles of Bis-Azo PC and 16 μ moles of DPPC: 5 μ moles of cholesterol: 1 μ moles of Bis-Azo PC fluctuates as the cholesterol concentration increases. In terms of zeta potential, all the SUV formulations showed the negative zeta potential. However, the difference is not statistically significant.

In general, there was significant difference ($P < 0.05$, ANOVA) in the size of SUV when comparing among all the formulations (Fig 3.8). It should be noted that SUV exhibited from 16 μ moles of DPPC and 16 μ moles of DPPC: 1 μ moles of Bis-Azo PC were non-significant. Similarly, there was no significant difference in zeta potential among all the SUV formulations. Hence the substitution of cholesterol into DPPC:Bis-Azo PC based SUV had significant effect on the size of vesicles.

Overall, it can be seen that the amount of cholesterol has a major impact on SUV formation using sonication. Furthermore, MLV formulation without cholesterol is easily converted into SUV, whereas MLV formulation with cholesterol takes more time in comparison to MLV formulation without cholesterol. In comparison to probe sonication, bath sonication is an ideal choice for SUV formation as it avoids the contamination of titanium particles (Barenholz and Lichtenberg, 2009) and circumvents the centrifugation step. Bath sonication has several advantages, such as, the sonicator being favourable for all sample sizes, with little risk of cross contamination and easier

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control of the lipid dispersion temperature. However, bath sonication has very high energy requirement compared to probe sonication. Even though probe sonication has faster processing time, it necessitates the use of centrifugation for at least three times, in order to make sure that all titanium particles are removed. Therefore, bath sonication was chosen for further IFV formation.

3.4.2. Effect of Interdigitation fusion vesicles content on vesicle size, zeta potential and protein entrapment efficiency

Initial studies show that calcium entrapment efficiency of MLV and SUV was smaller than IFV (Smith et al., 2007). As a result, effect of IFV contents on vesicle size, zeta potential and protein entrapment was validated in this study. The particle size, zeta potential and loading efficiency of the different IFV that were investigated in this study are shown in (Table 3.3).

3.4.2.1. Effect of vesicles lipid content on vesicle size

The average particle size and size distribution of vesicles are vital parameters for vesicles intended for drug delivery, for instance, inhalation, parental drug delivery and cancer drug delivery (Juliano and Stamp, 1975; Juliano, 1981). In 1991, (Komatsu and Rowe, 1991) demonstrated that lipid content of liposomes and inclusion of cholesterol inhibits bilayer interdigitation. Thereafter, Ahl and co-workers (Ahl et al., 1994) suggested that interdigitation fusion process is very sensitive to the addition of cholesterol or the lipids that resist the interdigitation. As cholesterol concentration increases then there is reduction in the bilayer interdigitation. Therefore, vesicles content is the important parameter in case of IFV formulation. With the intention to investigate the effect of IFV content on vesicle size, varying amounts of cholesterol were used in liposomes (DPPC: Bis-Azo PC). The addition of cholesterol to IFV was found to significantly ($p < 0.05$) increase the size of MLV, SUV and IFV (Table 3.3). SUV results are in line with those of (Friskén et al., 1999) and (Li-Ping Tseng, 2007). IFV prepared using DPPC:Chol:Bis-Azo PC (16:5:1) had mean diameter of about 15 μm whereas IFV prepared using DPPC:Chol:Bis-Azo PC (16:2:1) and DPPC:Chol (16:2) had mean diameters of 12 μm and 11 μm , respectively (Table 3.3). Similar results were obtained with MLV and SUV. The changes in size seen in all formulations suggest that incorporation of cholesterol may make DPPC bilayers more rigid. This is due to the cholesterol orientation behaviour into a phospholipid bilayer (Yeagle, 1985). Theoretically, within phospholipid bilayer, cholesterol upturns the degree of orientational order with its polar hydroxyl group and diminishes the rate of motion of the phospholipid hydrocarbon chains (Stockton and Smith, 1976; Yeagle et al., 1977). Furthermore, this process will produce a laterally more condensed membrane with increased packing density of the phospholipids (Lund-Katz et al., 1988; Smaby et al., 1994). That will increase the rigidity and decrease the permeability of the membrane (Needham and Nunn, 1990).

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Table 3.3 Effect of Interdigitation fusion vesicles content on vesicle size, zeta potential

MLV				SUV			IFV		
Formulation	Size (μm)	(PDI)	ZP (mV)	Size (μm)	(PDI)	ZP (mV)	Size (μm)	(PDI)	ZP (mV)
DPPC (16) μmoles	5.93± 0.01	1.72±0.01	-5.48 ± 6.5	0.11±10.7	0.370	-6.34 ± 3.5	7.20 ± 0.6	6.88±0.7	-5.48 ± 6.58
DPPC:Bis-Azo PC (16:1) μmoles	8.54± 0.20	6.25±0.16	-8.01 ± 0.7	0.10±9.6	0.286	-5.87 ±2.8	7.64± 0.8	3.94±0.2	-8.41 ± 0.70
DPPC:Chol (16:2) μmoles	8.49± 0.25	2.71±0.10	-4.54 ± 4.8	0.12±9.6	0.328	-2.98 ±2.4	11.4 ± 0.6	2.37±0.0	-4.54 ± 4.82
DPPC:Chol:Bis-Azo PC (16:2:1) μmoles	9.61± 0.77	3.90±0.31	-5.05 ± 2.5	0.15±6.1	0.295	-5.48 ± 6.5	12.4 ± 0.1	2.28±0.0	-5.65± 1.08
DPPC:Chol:Bis-Azo PC (16:4:1) μmoles	10.15± 0.34	2.00±0.04	-8.58 ± 2.5	0.17±5.9	0.311	-9.83 ±5.1	14.6 ±0.7	3.62±0.9	-8.36 ± 1.64
DPPC:Chol:Bis-Azo PC (16:5:1) μmoles	10.74± 0.33	2.14±0.05	-7.78 ±3.0	0.18±2.2	0.276	-6.21 ±1.01	15.55 ± 0.2	2.45±0.01	-7.47 ±1.85

IFV: Liposomes composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC, where applicable. All formulations were prepared by the interdigitation fusion method entrapping the BSA-FITC. Vesicle size and zeta potential of liposomes was measured in double-distilled water and 0.001 M PBS, respectively, at 25 °C using Sympatec and Zeta Plus. Results denote the mean ± SD from at least 3 independent batches.

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Despite of changes in size, the polydispersity of all liposomes remained unchanged. However, it should be noted that polydispersity was expressed as polydispersity Index (PDI). The results in this study mirrors several other studies that have suggested that cholesterol structure is very different from other lipids because of its flat, rigid fused ring structure, enabling cholesterol to develop more stable stacked aggregates compared to other lipids (Epand et al., 2003). Earlier researcher (Lee, 1977) and (Nayar et al., 1989) verified that in the absence of cholesterol homogenous LUV composed of long-chain phosphatidylcholines generate packing defects upon cooling below to their phase transition temperatures. In contrast, other studies (Demel et al., 1972; Corvera et al., 1992) demonstrated that the addition of higher amounts of cholesterol in liposomes increased the packing densities of phospholipids and reduced the bilayer permeability to drugs. Similarly, results from this study (Table 3.3) suggest that at smaller concentrations of cholesterol produced stable liposomes. On the other hand, at higher concentrations, it increases the particle size and rigidity of liposomes.

It is also evident from Table 3.3 that the addition of photosensitive lipid Bis-Azo PC into the vesicles has neither effect on the size of MLV, SUV and IFV nor the polydispersity of Liposomes. Consequently, IFV prepared using DPPC had a similar particle size distribution with the IFV prepared using DPPC:Bis- Azo PC.

3.4.2.2. Effect of Interdigitation fusion vesicles content on zeta potential

The net surface charge of vesicles is an important parameter that will affect the electrostatic interaction between vesicles and surrounding molecules, particles and surfaces. In theory, it is constrained by the various parameters, such as, head group of lipid, pH and ionic strength of the dispersion medium. Liposomal stability relies on the magnitude of zeta potential. If all the particles in liposomes acquire a large negative or positive zeta potential then they will have a tendency to repel each other and that will avoid the aggregation (Paolino, 2006).

From Table 3.3, it is apparent that all liposomes show a slight negative zeta potential. It is well known that DPPC is a neutral lipid and possess a net neutral charge because at neutral pH, the head group of phospholipid always carries a both a negative zeta potential (at the phosphate group) and a positive zeta potential (at the choline group) and therefore does not possess a net charge. This is reflected in the results obtained in this study that show negative zeta potential. The outcome could possibly be due to two reasons:

1. Fisar already reported that over a wide range of pH values, phosphatidylcholine molecules are completely electroneutral as the negative charge of the phosphate group is compensated by the positive charge of the choline head (Fisar, 2005) even though, neutral PC shows negatively

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zeta potential. This is due to the hydration of the surface and the orientation of the charged elements of the lipids. Based on the pH, the head region of phospholipids is expected to be cations at pH values lesser than one, zwitterions over a large range of pH and probably anions at higher pH (Deb, 2001).

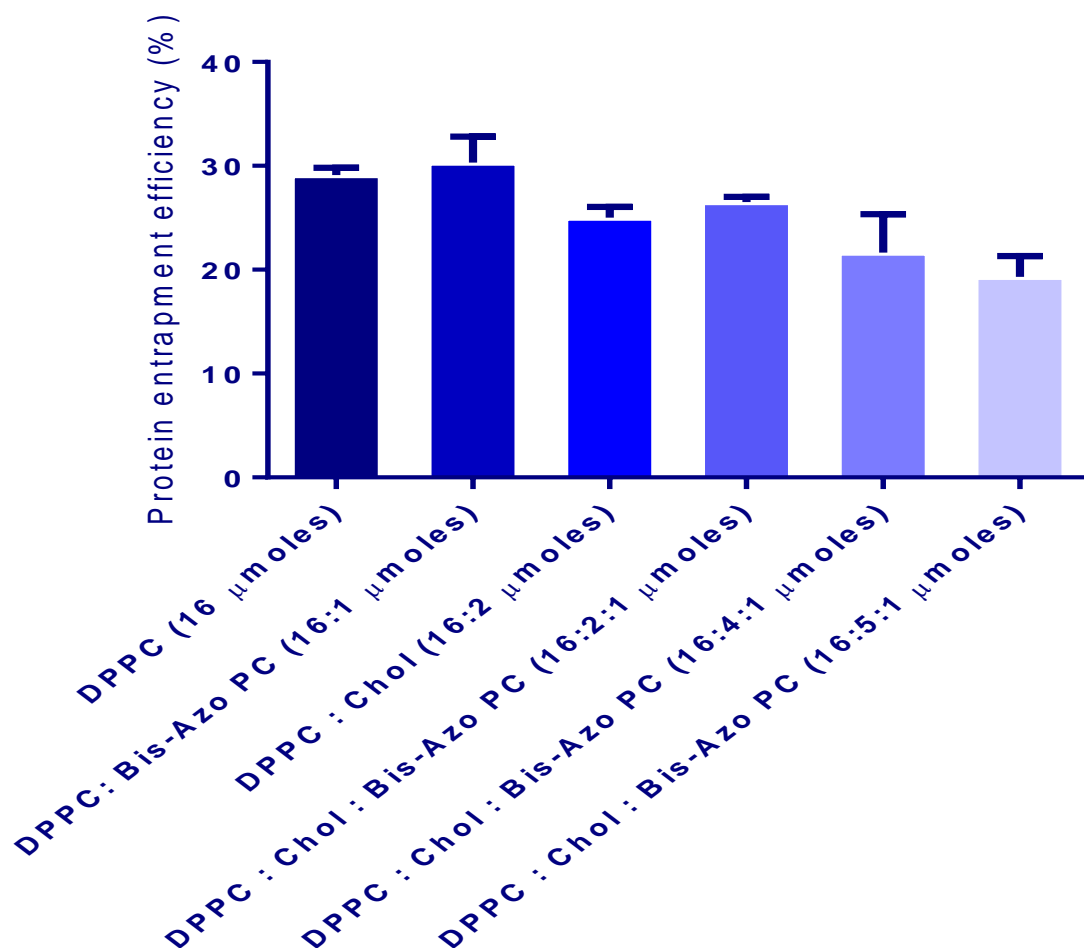
2. DPPC's shows negative zeta potential due to conformational changes in the lipid head group region of the liposomes (Law et al., 1988; Makino et al., 1991). In their lab study, they noted that the direction of the lipid head region of phospholipid is very sensitive to the temperature and the ionic strength of dispersion medium. According to their data, at a constant temperature, when the ionic strength of dispersion medium increased, the choline group proceeds towards the outer part of the bilayer surface, while the phosphatidyl group lies behind the bilayer surface. Moreover, at the phase transition temperature, the choline group lies in the inner most region whereas the phosphatidyl group is located in the outermost region.

Interestingly, some studies inferred that incorporation of cholesterol has little effect on head group separation in the liposomes made from the zwitterionic egg phosphatidylcholine (PC) and anionic egg phosphatidylglycerol (Yeagle et al., 1977). Their results clearly indicate that the inclusion of cholesterol up to 50 mole % in the bilayer produced null effect on the zeta potential of liposomes and eventually addition of sterols among the fatty acyl chains of phospholipids causes little or no displacement of the head group at the bilayer surface. Parallel results are also obtained in case of lanosterol and epicoprostanol, a sterol with a non-planar ring system (A/B cis) (Rottem et al., 1971). It is also clear from Table 3.3 that there are no changes in the zeta potential of MLV, SUV and LUV and that all the liposomes had parallel zeta potential. Therefore, we can say the inclusion of varying amounts of cholesterol has considerable effect on the zeta potential of DPPC liposomes which need further study.

3.4.2.3. Investigation of interdigitation fusion vesicles content on protein entrapment

To investigate protein entrapment within photo-sensitive liposomes, a protein labelled with fluorescent marker i.e. bovine serum albumin conjugated with fluorescein isothiocyanate (BSA-FITC) was used. BSA-FITC was selected as a model protein and has been studied in terms of interaction with liposomes. Preliminary results demonstrated the influence of bilayer composition on BSA-FITC loading (Fig 3.9).

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Formulations

Fig 3.9: Effect of IFV contents on protein entrapment.

IFV formulations were prepared by the interdigitation fusion method entrapping the BSA-FITC. The entrapment efficiency of BSA-FITC was determined by using fluorescence spectroscopy. Result represents mean \pm SD, $n=3$.

As can be seen from Table 3.3 and Fig 3.9, IFV prepared using DPPC and DPPC:Bis-Azo PC had a higher protein loading efficiency compared with IFV prepared using DPPC:Chol and DPPC:Chol:Bis-Azo PC. However, there was no any significant difference seen between the IFV formulations. The protein loading efficiency of IFV prepared using DPPC and DPPC:Bis-Azo PC was found to be 29 % and 30 % respectively. These results are similar with previously published

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data by others (Pick, 1981; Ohsawa et al., 1984; Liu and Yonetani, 1994) who prepared LUV using different method i.e. freeze- thaw method.

Studies by the above named researchers (Pick, 1981; Ohsawa et al., 1984; Liu and Yonetani, 1994), using LUV for the encapsulation of protein drugs or solutes, have shown entrapment levels of up to from 20 to 30 %, similar to levels observed in the results in Fig 3.9. However, in these studies the freeze-thaw method was used, which involves formation of SUV from MLV which is then rapidly frozen and followed by slow thawing. Owing to this rapid freezing and thawing process, SUV fuse with each other and these aggregates get converted into LUV. Ideally, this technique promotes higher drug loading because of morphological changes, which occur during the process of freezing and thawing of the liposomes and thus resulting in increased aqueous volumes of liposomes (Mayer et al., 1989). Other techniques available for the formulation of LUV include the reverse phase evaporation method; here LUV are generated from water-in-oil emulsions using buffers and excess organic phase, under reduced pressure (Szoka and Papahadjopoulos, 1978). LUV prepared from this method possess higher aqueous-to-lipid volume ratio and entrap larger percentage of hydrophilic drugs. Earlier, LUV are prepared by extrusion method (Hope et al., 1985) in which the rapid production of LUV are carried out by repeated extrusion under moderate pressure (≤ 500 lb/in²) of MLV through polycarbonate filters (100 nm pore size).

From Fig 3.9, it is evident that the photosensitive lipid Bis-Azo PC has no effect on protein loading efficiency as the protein loading efficiency of DPPC was 29 % and DPPC:Bis-Azo PC was 30 %, although 1 μ moles of Bis-Azo PC was used. However, it should be noted that inclusion of varying amounts of cholesterol reduces the protein loading efficiency of IFV. This may be a result of increased packing densities of phospholipids (Semple et al., 2005). To ascertain whether this was indeed surface pressure measurement a study was done by (Rogerson et al., 1987) which clearly show a decrease in effective area per molecule as the cholesterol content of the monolayer is increased.

Earlier investigations on examining the effect of liposome composition on drug entrapment (Gregoriadis and Davis, 1979a) established that addition of 50 % mol/mol cholesterol within a liposome formulation not only increased the stability, but also reduced the permeability of bilayers. In terms of photosensitive liposomes, it was revealed (Bisby et al., 1999b) that inclusion of varying amounts of cholesterol (up to 25 mol %) within the bilayers increased the visible light sensitivity of photosensitive liposomes. On the other hand, supplementation of DPPC:Bis-Azo PC bilayer with 2 μ moles of cholesterol resulted in a reduction in protein loading from 30 % to 26 % (Fig 3.9). Furthermore, increasing cholesterol amount leads to reduced protein loading efficiency i.e. with 4 μ moles and 5 μ moles of cholesterol, protein loading efficiency was reduced to 21 % and 19 %, respectively.

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respectively (Fig 3.9). It would appear that with different formulations the protein loading tend to be affected only marginally i.e. 2-4 %.

This cholesterol –induced effect on protein loading in IFV was consistent with other results (Smith et al., 2007) (Ahl et al., 1994) that proved that the cholesterol hampered the formation of IFV by making obstacle to the formation of the interdigitated lipid sheets. Conversely, it is recognised that inclusion of cholesterol increased the stability of liposomes (Gregoriadis, 1993) and triggered wavelengths in blue region of visible the spectrum (> 400 nm). As a result, the presence of cholesterol in the SUV liposomes used as precursors for IFV may possibly result in liposomes with higher stability. However, limiting the appropriate interdigitation process ultimately inhibits the production of IFV with higher entrapment. This fact was further confirmed by the increment in vesicle size for IFV containing 4 μ moles and 5 μ moles of cholesterol with sizes 14 μ m and 15 μ m compared with ≤ 7 μ m for the cholesterol free DPPC liposomes (Table 3.3) and (Fig 3.9).

Data shown in Table 3.3 and Fig 3.9 indicates that incorporation of cholesterol may make DPPC bilayers more rigid which further reduced the protein entrapment efficiency. This rigidity could be the reason for increment in the particle size of liposomes with the varying amounts of cholesterol. According to (Bernsdorff et al., 1997; Mohammed et al., 2004b), hydrophobic interaction could be another reason due to which entrapment efficiency of IFV was decreased. Therefore entrapment efficiency of IFV prepared using 4 μ moles and 5 μ moles of cholesterol was 21 % and 19 %, respectively. Smith and co-workers (Smith et al., 2007) also revealed that entrapment efficiency of photosensitive IFV decreased with increased concentration of cholesterol.

3.4.3. Confocal microscopy

One of objective of this study was to visualize the morphology of vesicles and to check whether BSA-FITC gets entrapped within well-formed vesicles using light microscopy. In this study, vesicles with or without entrapped protein marker, BSA-FITC, were prepared using formulations of DPPC:Chol:Bis-Azo PC (16:2:1 μ moles) and visualized on a Leica multiphoton confocal microscope using an 63 X oil objective. Fig 3.10 illustrates the MLV, SUV and IFV without BSA-FITC and Fig 3.11 represents the MLV, SUV and IFV with BSA-FITC.

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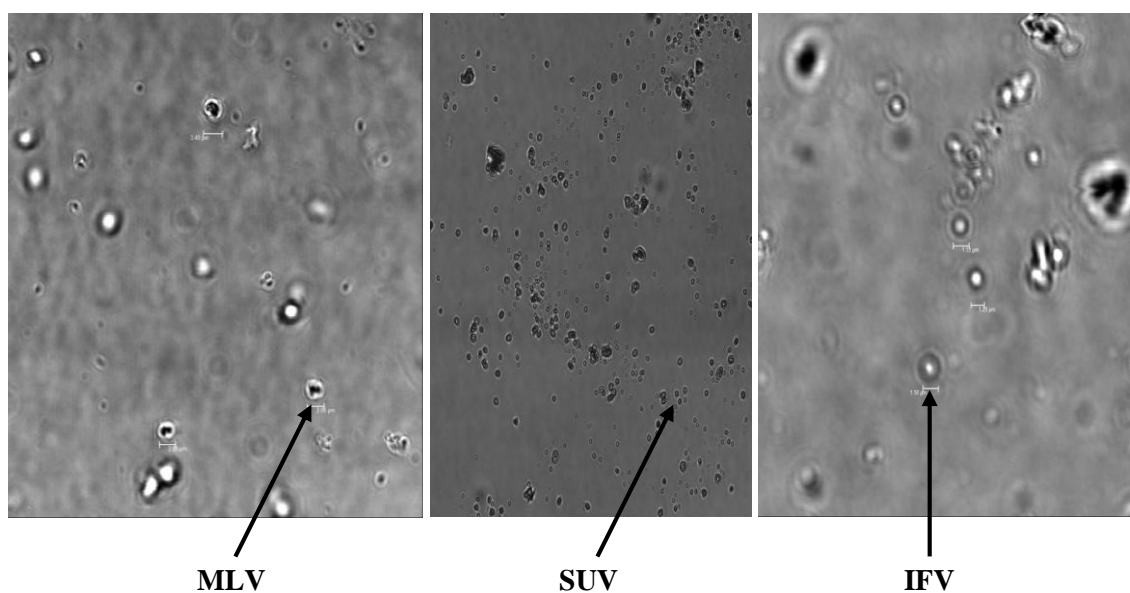


Fig 3.10: Confocal microscopy images of DPPC:Chol:Bis-Azo PC at 16:2:1 μ moles liposomes (MLV, SUV and IFV) without fluorescent protein taken with a multiphoton Confocal Microscope using a 63 X objective.

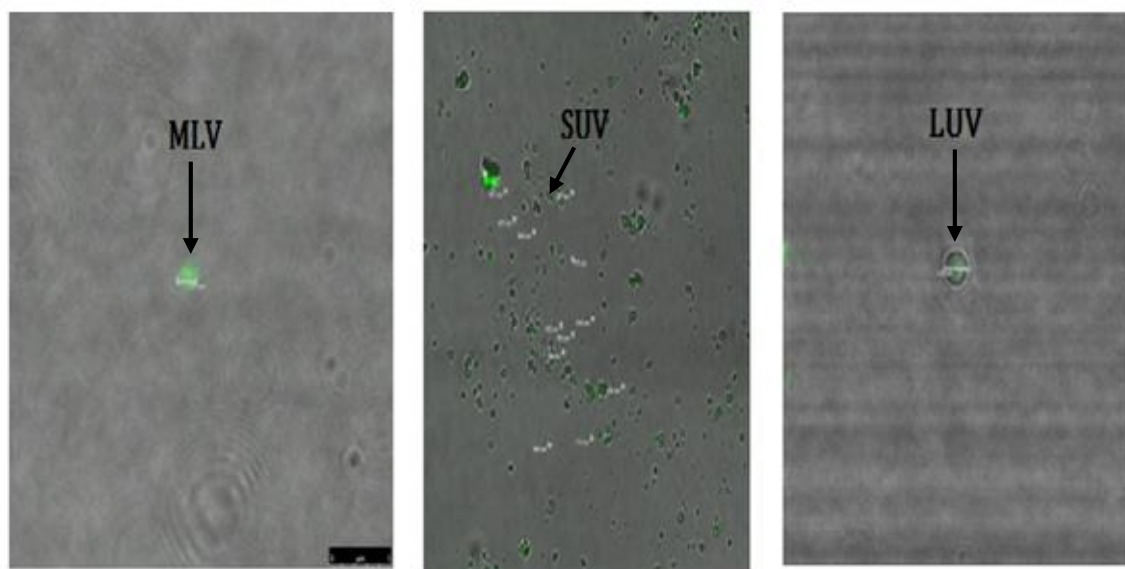


Fig 3.11: Confocal microscopy images of DPPC:Chol:Bis-Azo PC at 16:2:1 μ moles liposomes (MLV, SUV and IFV) entrapped with BSA-FITC taken with a multiphoton Confocal Microscope using a 63 X objective.

The green fluorescent marker shown is BSA-FITC and is present inside the liposomes i.e. internal aqueous compartment of liposomes.

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It is revealed from the images (Fig 3.10 and Fig 3.11) that the IFV were prepared using interdigitated fusion method. Moreover, diameter of IFV is greater than SUV and smaller than MLV. Furthermore, Fig 3.11 also evidences that the BSA-FITC can be seen entrapped within the internal aqueous core of all the vesicles.

3.4.4. Photo-induced protein release

Morgan and co-workers (Morgan et al., 1995) previously demonstrated that photosensitive liposomes containing Bis- Azo PC required only a few minutes for total release of the drug from the liposomes and the similar liposomes cease to trap calcein when the Bis-Azo PC content exceeds about 8 % (mol:mol) of the total lipid content of Bis-Azo PC and dipalmitoyl-1- α -phosphatidylcholine (DPPC). Later on, (Bisby et al., 1999a) revealed that effective photo-induced calcein release and better stability was achieved for liposomal composition containing 6 % (mol:mol) of Bis-Azo PC in the DPPC. Therefore, smaller amount of concentrations of photosensitive lipid i.e. Bis-Azo PC were used in subsequent studies reported within this chapter. BSA-FITC leakage from liposomes prepared from DPPC; DPPC:Chol; and DPPC with varying levels of cholesterol and containing a fixed level of 1 μ moles of Bis-Azo PC is shown in Table 3.4 and Fig 3.12. Each formulation tested for protein release using 3 different UV lamp systems: a UV curing flood lamp, a ROHS 36 w professional UV lamp and a Nichia NSHU590E UV LED lamp with emission centered at 365 nm.

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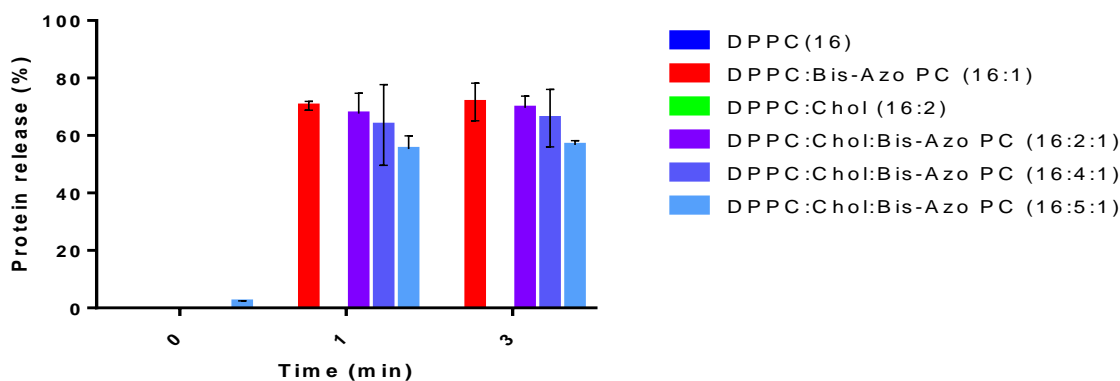
Table 3.4: Photo-induced protein releases from liposomes

Formulation	Initial drug loading (%)	No light (% released)	UV exposure time	Lamp 1 (%released)	Lamp 2 (%released)	Lamp 3 (%released)
DPPC (16) μ moles	29.10 \pm 0.7	0%	1 min	0%	0%	0%
	29.10 \pm 0.7	0%	3 min	0%	0%	0%
DPPC:Bis-Azo PC (16:1) μ moles	30.33 \pm 2.51	0%	1 min	70.33 \pm 1.52	73.56 \pm 5.13	39.2 \pm 3.70
	30.33 \pm 2.51	0%	3 min	71.66 \pm 6.50	78 \pm 8.02	40.33 \pm 5.50
DPPC:Chol (16:2) μ moles	25 \pm 1.05	0%	1 min	0%	0%	0%
	25 \pm 1.05	0%	3 min	0%	0%	0%
DPPC:Chol:Bis-Azo PC (16:2:1) μ moles	26.5 \pm 0.5	0%	1 min	67.66 \pm 7.02	70.66 \pm 8.02	31.33 \pm 1.52
	26.5 \pm 0.5	0%	3 min	69.16 \pm 4.04	70.33 \pm 6.50	33.56 \pm 13.8
DPPC:Chol:Bis-Azo PC (16:4:1) μ moles	21.66 \pm 3.78	0%	1 min	63.86 \pm 14.1	65.33 \pm 3.51	25 \pm 5.56
	21.66 \pm 3.78	0%	3 min	66.01 \pm 10.0	70.33 \pm 7.50	31.66 \pm 3.05
DPPC:Chol:Bis-Azo PC (16:5:1)	19.33 \pm 2.08	2%	1 min	55.33 \pm 4.50	58 \pm 6.55	18 \pm 6.01
	19.33 \pm 2.08	2%	3 min	56.66 \pm 1.52	59.26 \pm 1.78	22.33 \pm 6.50

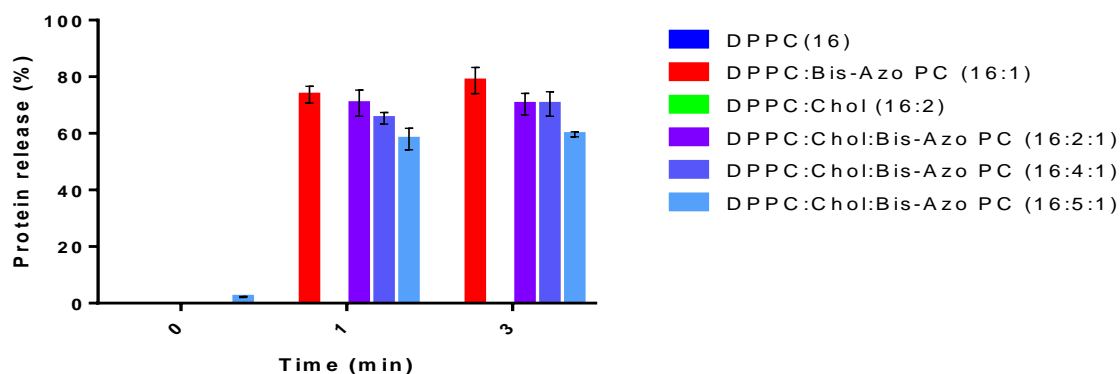
Photosensitive and non-photosensitive IFV: Photosensitive and non-photosensitive IFV were prepared via interdigitation fusion method. IFV composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC, where applicable. Photo-trigger release studies were done by using three different UV lamps. The percentage releases of BSA-FITC were measured using fluorescence spectroscopy. The data are expressed as means \pm standard deviation of three independent batches.

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a)



b)



c)

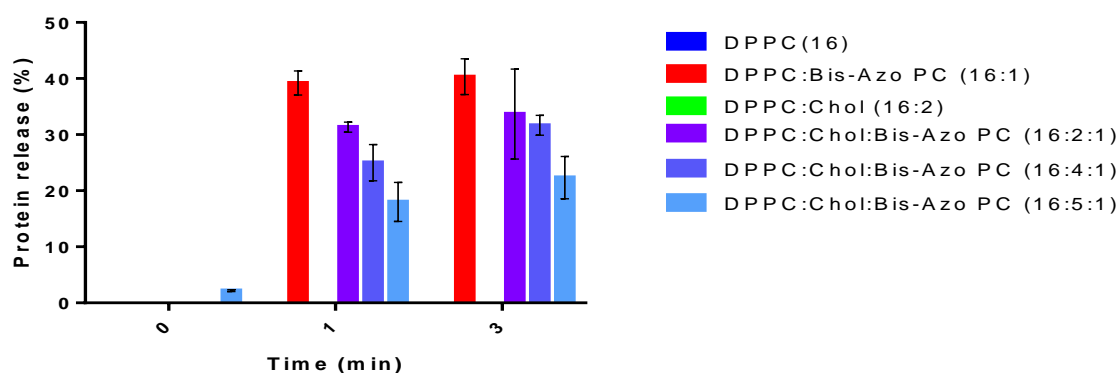


Fig 3.12: Photo-induced protein release by using three different UV lamp: a) UV curing flood lamp b) ROHS 36 w professional UV lamp and c) Nichia NSHU590E UV LED lamp.

Only 4 bars are visible in the following graphs but with six samples. Bars of DPPC(16) and DPPC:Chol(16:2) overlap each other due to 0 % release.

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Table 3.5: Summary of measured intensity of UV lamps used for this investigation

Lamp number	UV lamp	Power intensity (I) mW/cm ²
1	UV curing flood lamp	1.81 x 20 mW/cm ²
2	ROHS 36 w professional UV lamp	2.94 x 20 mW/cm ²
3	Nichia NSHU590E UV LED lamp	0.74 x 20 mW/cm ²

For photo-trigger release study, three different UV lamps were used. The emission characteristics of all these lamps were measured by Accucal 50 UV intensity meter.

From Table 3.5, it can be seen that the measured intensity of lamp 1 and lamp 2 is $I = 1.81 \times 20$ mW/cm² and $I = 2.94 \times 20$ mW/cm², respectively, which is greater than the measured intensity of lamp 3 ($I = 0.74 \times 20$ mW/cm²). Hence, due to power difference lamp 1 and lamp 2 showed better protein release than lamp 3 (Fig 3.12).

As can be seen from Fig 3.12, prior to exposure to UV, no protein release was detectable ; however, after UV exposure the three different lamps gave different patterns of release of fluorescent protein from the liposomes, yet with all three lamps the presence of photosensitive lipid Bis-Azo PC in the DPPC liposomes promotes a triggered release of the protein. This is supported by observation of control samples, in which only DPPC and DPPC along with the 2 μ moles of cholesterol are used (Fig 3.12). These formulations are not sensitive to the blue light at 365 nm and thus no release of BSA-FITC is seen after UV irradiation at 1 min and 3 min. In contrast, DPPC:Bis-Azo PC (16:1) based IFV showed 73 % and 71 % release after UV irradiation (ROHS 36 w professional UV lamp) at 1 min and 3 min. These results are consistent with results reported by Bisby et al. (Bisby et al., 1999b) who also suggested that threshold concentration of photosensitive lipid Bis-Azo PC is required for the photo-induced release of solutes in response to laser light. According to their study, Bis-Azo PC showed photo-isomerization with laser irradiation and two types of effects could be observed in case of laser-induced photo-isomerization. Firstly, there might be a rapid perturbation of the liposomal membrane tempted by the volume expansion from the trans- form of the lipid (compacted structure) to the cis-form of the lipid (expanded structure). Consequently, there might be a time-dependent relation phase in which bulky, polar, cis

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form of lipid, rearranges to an equilibrium state i.e. lateral redistribution of isomerized lipids and entrapped material is released. They also noted that during laser pulse experiment, small reproducible lag phase is obtained before the release of solutes and which is correlated with the lateral redistribution of Bis-Azo PC. The data obtained from our experiments (Fig 3.12) also gave the same conclusion as burst release of BSA-FITC obtained due to the presence of Bis-Azo PC.

Statistical analyses ($P < 0.05$) from Fig 3.13 shows that the difference between values of protein release from photosensitive liposomes due to lamp 2 vs lamp 1 was statistically significant ($P < 0.01$), lamp 2 vs lamp 3 was very significant ($P < 0.001$) and lamp 1 vs lamp 3 was very significant ($P < 0.001$).

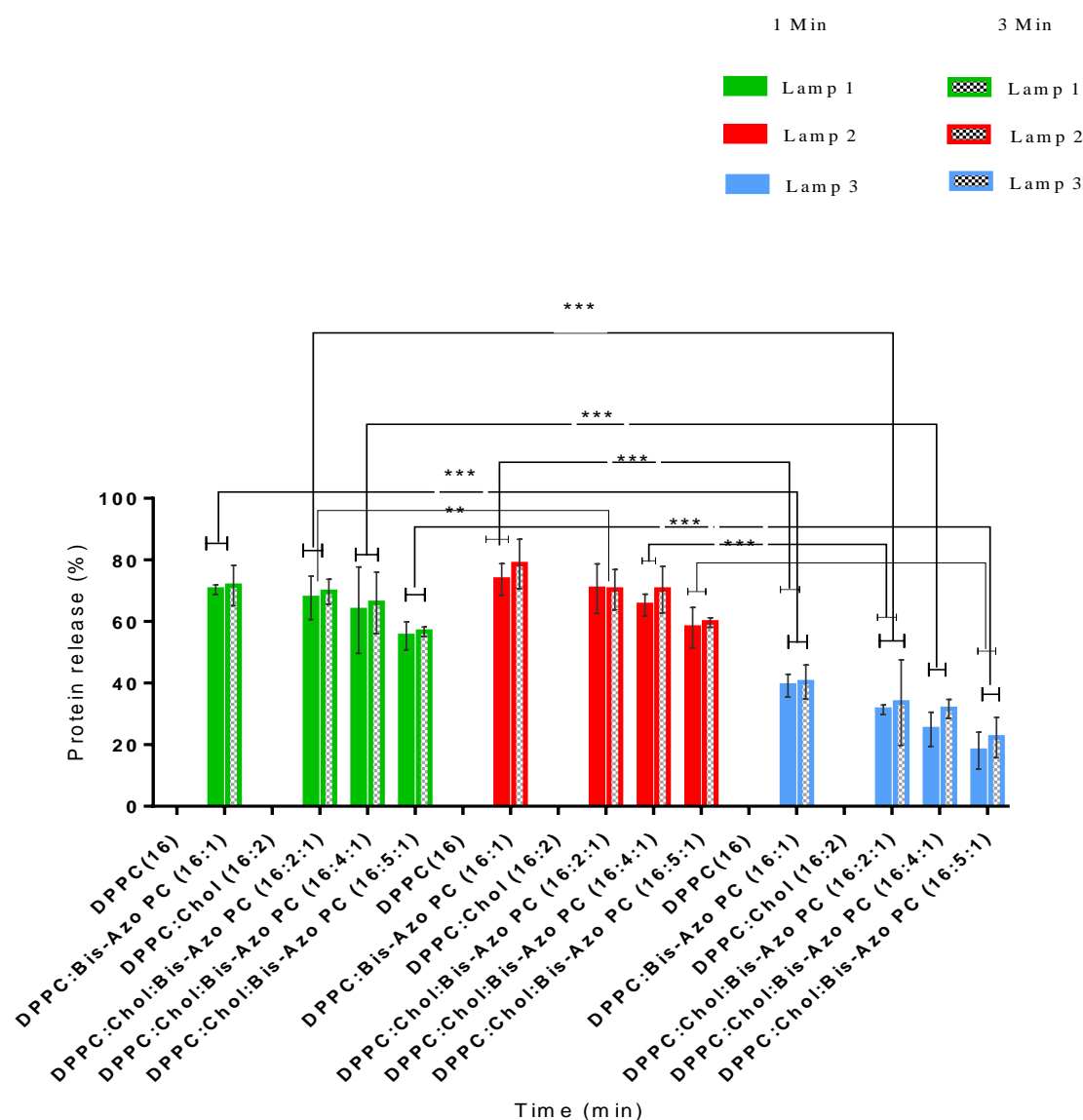


Fig 3.13: Summary of ANOVA analysis.

All the protein release data in Fig 3.12 passed the normality test using one-way analysis of variance (ANOVA) and a multiple comparison post-test Tukey.

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Another interesting difference was observed between photosensitive liposomes prepared from DPPC:Bis-Azo PC with varying amount of cholesterol (Fig 3.13). The protein release pattern from photosensitive liposomes was significantly ($P < 0.001$) reduced as the amount of cholesterol is increased. From Fig 3.13, it is also clear that photosensitive liposomes containing DPPC:Chol:Bis-Azo PC (16:2:1) have significantly ($P < 0.001$) higher release of BSA-FITC in comparison with other photosensitive liposomes containing DPPC:Chol:Bis-Azo PC (16:4:1) and DPPC:Chol:Bis-Azo PC (16:5:1). With lamp 2 UV irradiation, photosensitive liposomes containing DPPC:Chol:Bis-Azo PC (16:2:1) gave 70 % protein release after 1 min. On the other hand, photosensitive liposomes containing DPPC:Chol:Bis-Azo PC (16:4:1) and DPPC:Chol:Bis-Azo PC (16:5:1) gave 65 % and 58 % protein release after 1 min. Similar trend of the protein release was obtained after 3 min UV irradiation using the same lamp and the same liposomal formulations. Therefore, the inclusion of 4 and 5 μ moles of cholesterol had a dramatic effect on protein leakage in this system. This major influence of cholesterol on the properties of these liposomal formulations evidenced a possible way to control their permeability. Previous work (Morgan et al., 1987a) has shown that cholesterol has a major impact on the percentage release of solute from photosensitive liposomes containing Bis-Azo PC and a single azo benzene moiety known as “Pazo PC”. According to them, Cholesterol is very complex in nature and well recognised to act as ‘buffer’ of membrane order. In fluid phase, cholesterol increases the order and packing density within the bilayer, whereas in the gel phase, the order and packing density within the bilayer is decreased (Bisby et al., 1999c). As a result, cholesterol has two noteworthy effects, primarily cholesterol affects the lateral phase separation and subsequently it alters the overall rigidity of the lipid bilayer (Ohvo-Rekilä et al., 2002). Both of these two effects will influence the kinetics of solute release from photosensitive liposomes. Later on Xu and London (Xu and London, 2000) demonstrated how cholesterol induces lipid domain formation and considered sterols which do not induce lipid domain as anti-cholesterols. They also evidenced that cholesterol promotes tight packing of saturated lipids which promote domain formation and this lipid domain formation plays a key role in lateral phase separation. However, the promising role of cholesterol in phase separation and the photo-induced release process needs further investigation. Analytical methods such as Differential scanning calorimetric (DSC) measurements, freeze-fracture electron microscopy, and NMR and electron spin resonance (ESR) spectroscopy would be beneficial in this respect.

A BSA-FITC release of 2 %, without the use of UV irradiation, from photosensitive liposomes containing DPPC:Chol:Bis-Azo PC (16:5:1) was noted (Table 3.4). Conversely, all other liposomes showed 0 % release without UV irradiation. Liposomes containing higher amounts of cholesterol and photosensitive lipid Bis-Azo PC have been noted to give a trigger release without UV irradiation (Bisby et al., 2000b). The reason behind this leakage is that cholesterol containing

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liposomes allow stepped partial release of entrapped solute, following multiple periods of short visible illumination.

Overall, results presented in Fig 3.12 demonstrated that release of BSA-FITC was achieved with very low exposure to UV light. This protein release during UV irradiation is likely due to the photo-isomerisation (trans-cis isomerisation) of Bis-Azo PC (Bisby et al., 1999a). It should be noted that photosensitive lipid, Bis-Azo PC, can exist in several forms, with one or both chains in the trans- form. During photo-isomerisation, trans-form of Bis-Azo PC is converted to cis-form allowing the entrapped content to be released. For that reason, IFV containing photosensitive lipid Bis-Azo PC, showed the release of protein after UV irradiation, whereas IFV without Bis-Azo PC showed 0 % release after UV irradiation, for instance, DPPC (16) and DPPC:Chol (16:2), (Fig 3.12).

From this study, photosensitive liposomes of DPPC:Chol:Bis-Azo PC (16:2:1) seem to be the optimal liposome formulation for the controlled drug release under a UV light. Apart from this formulation, IFV prepared using DPPC:Chol:Bis-Azo PC (16:4:1) is also good for the photo-induced drug release. However, it is evidenced that the protein loading efficiency of this formulation is low (21 %), in comparison with protein loading efficiency of DPPC:Chol:Bis-Azo PC (16:2:1) which is 26 % (Fig 3.9). Therefore, IFV prepared using DPPC:Chol:Bis-Azo PC (16:2:1) was used for further investigation. For UV irradiation, lamp 2 (ROHS 36 w professional UV lamp) (Fig 3.6) was selected as the best choice for further study, as lamp 1 and lamp 3 gave less protein release from photosensitive IFV (Fig 3.12).

The amount of cholesterol in photosensitive liposomes has a major influence in protein release. At low concentrations, cholesterol gives stability to the liposomal bilayer and show burst release of entrapped materials after UV irradiation. On the other hand, at higher concentration, Cholesterol gives rigidity to the bilayer and reduces the release of entrapped materials after UV irradiation. As can be seen from Fig 3.12, photosensitive IFV prepared using small concentrations of cholesterol, DPPC:Chol:Bis-Azo PC (16:2:1), gave the burst release of protein after 1 and 3 min of irradiation. In contrast, photosensitive IFV prepared using higher concentrations of cholesterol, DPPC:Chol:Bis-Azo PC (16:5:1), gave smaller release of protein after 1 and 3 min. Furthermore, this photosensitive IFV released 2 % of protein without the use of UV irradiation. Hence, the photosensitive IFV prepared using higher amounts of cholesterol have reduced stability.

3.4.5. Investigation of the 100 % of protein release from DPPC IFV using ROHS 36 w professional UV curing lamp.

Although photosensitive IFV formulations containing BSA-FITC were found to be photo active by using three different lamps, the most important question was whether they would give 100 %

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release of protein or not. Therefore, based on the photo-induced release data described in section 3.4.3, similar IFV formulations were selected for this experiment. The formulations were irradiated through, using lamp 2 (ROHS 36 w professional UV curing lamp) for different time intervals i.e. 0, 1, 3, 6, 9, 12 and 15 min. It should be noted that for this study, lamp 2, which was used because of showing better release in comparison to lamps 1 and 3. The data obtained from this investigation are presented in Fig 3.14.

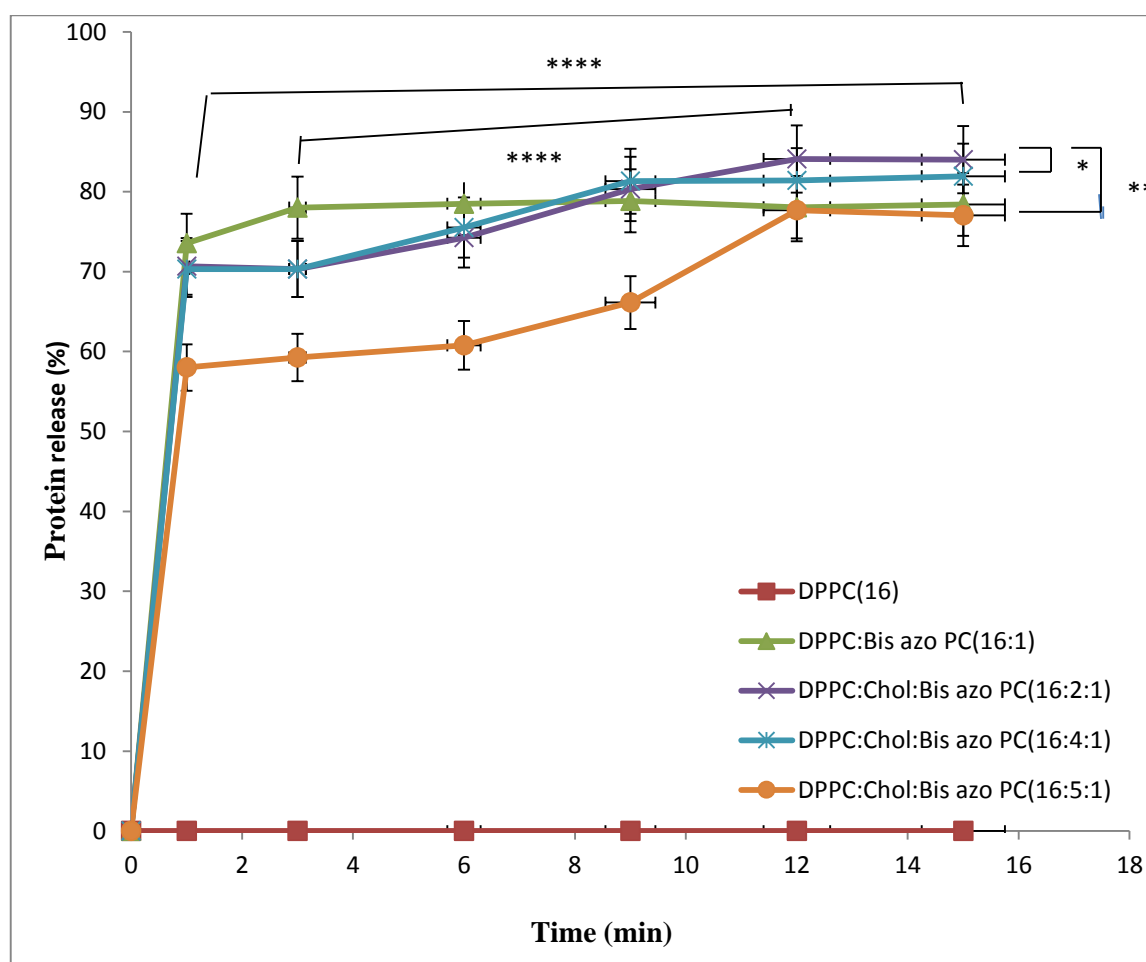


Fig 3.14: Photo-triggered release of protein from IFV with ROHS 36w professional UV curing lamp:

IFV were prepared via interdigitation fusion method. IFV composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC, where applicable. The 100 % releases of BSA-FITC were achieved with lamp 2 by measuring fluorescence emission. The data are expressed as means \pm standard deviation of three independent experiments. Significance was measured by one-way ANOVA (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

Fig 3.14 clearly indicates that there is no further protein release from the IFV, after 12 min of irradiation. A plot of cumulative percentage of drug release vs irradiation time, revealed protein release patterns of all IFV formulations except DPPC and DPPC:Chol, whose release are slow but increases with increased UV exposure time.

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It is also clear from Fig. 3.14 that after 12 min of UV irradiation, IFV prepared using DPPC:Chol:Bis-Azo PC (16:2:1) shows maximum protein release of up to 84 % as compared to other IFV. Indeed, IFV prepared using DPPC:Chol:Bis-Azo PC (16:5:1) shows minimum protein release, of up to 77 %, while, IFV prepared using DPPC:Chol:Bis-Azo PC (16:4:1) shows protein release of up to 81 %. These results demonstrate that the inclusion of very high amounts of cholesterol reduces the % protein release. Interestingly, control experiments, in which IFV formulations prepared using DPPC and DPPC:Chol (16:2) show 0 % release after prolonged exposure to UV irradiation. This further cements the hypothesis that the presence of photosensitive lipid Bis-Azo PC into the phospholipid causes disruption and releases the entrapped solutes (Bisby et al., 1999b).

Statistical analyses ($P < 0.05$) shows that the % of protein release from IFV after 1 and 3 mins of UV exposure compared to % of protein release after 12 min and 15 min, as statistically significant ($P < 0.0001$) (Fig 3.14). It was also noted that the % of protein release difference between DPPC:Chol:Bis-Azo PC (16:2:1) vs DPPC:Chol:Bis-Azo PC (16:4:1) was statistically significant ($P < 0.05$), while, DPPC:Chol:Bis-Azo PC (16:4:1) vs DPPC:Chol:Bis-Azo PC (16:5:1) and DPPC:Chol:Bis-Azo PC (16:2:1) vs DPPC:Chol:Bis-Azo PC (16:5:1) was very significant, ($P < 0.01$) and ($P < 0.001$), respectively (Fig 3.14).

It is also revealed from Fig 3.14, that IFV prepared using DPPC:Bis-Azo PC (16:1) shows % protein release of up to 78 % after 3 min irradiation compared to other IFV. This may be possibly due to the presence of Bis-Azo PC, this formulation does not contain cholesterol, thus the fast release (Sandhu et al., 1986). Conversely, IFV prepared using the DPPC, Bis-Azo PC supplemented with 2 and 4 μ moles of cholesterol shows release of up to 70 % after 3 min, demonstrating the importance of cholesterol. From earlier experiments, Demel et al (Demel et al., 1972) and Papahadjopoulous (Papahadjopoulos et al., 1973a), established that cholesterol has the ability to complex with phospholipids and that this complex could reduce bilayer permeability to hydrophilic drugs or solutes. Apart from this, other researchers (Mohammed et al., 2004b; Sułkowski et al., 2005), had reported that addition of cholesterol into the liposome increases liposomes stability and reduces permeability of entrapped materials. In theory, Cholesterol combines with phospholipids and acts as a stabiliser.

It seems that 100 % protein release from photosensitive IFV was not achieved (Fig. 3.14) due to incomplete recovery of the added protein. Therefore, IFV prepared using DPPC:Chol:Bis-Azo PC (16:2:1) show high protein release with a maximum of 84 % after 12 min UV exposure. This formulation had cholesterol content of 2 μ moles which not only gives stability to the IFV, but also reduces the bilayer permeability. Other IFV formulations, such as DPPC:Chol:Bis-Azo PC showed

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protein release of upto 81 %. However, this formulation contains cholesterol up to 4 μ moles which increases rigidity and reduces protein entrapment efficiency.

3.4.6. Stability assessment of IFV formulations

In order to develop highly efficient photosensitive controlled release systems, it is necessary that the formulated IFV to have high retention of the loaded BSA-FITC when in a non-photoreactive environment; thus, a stability study was carried out at two different temperatures- 1) Short- term stability at room temperature ($\sim 23^{\circ}\text{C}$) and 2) Long-term stability at 4°C .

3.4.6.1. Short-term stability

In this stability study, IFV formulations of DPPC 16 μ moles, DPPC:Bis-Azo PC 16:1 μ moles, DPPC:Chol:Bis-Azo PC 16:2:1 μ moles, DPPC:Chol:Bis-Azo PC 16:4:1 μ moles, DPPC:Chol:Bis-Azo PC 16:5:1 μ moles were stored at room temperature ($\sim 25^{\circ}\text{C}$) for up to 15 min, 24 h and 48 h, respectively and assessed in terms of variation in size, zeta potential, span and drug retention inside the liposomes. The results are presented in Figs 3.15, 3.16, 3.17.

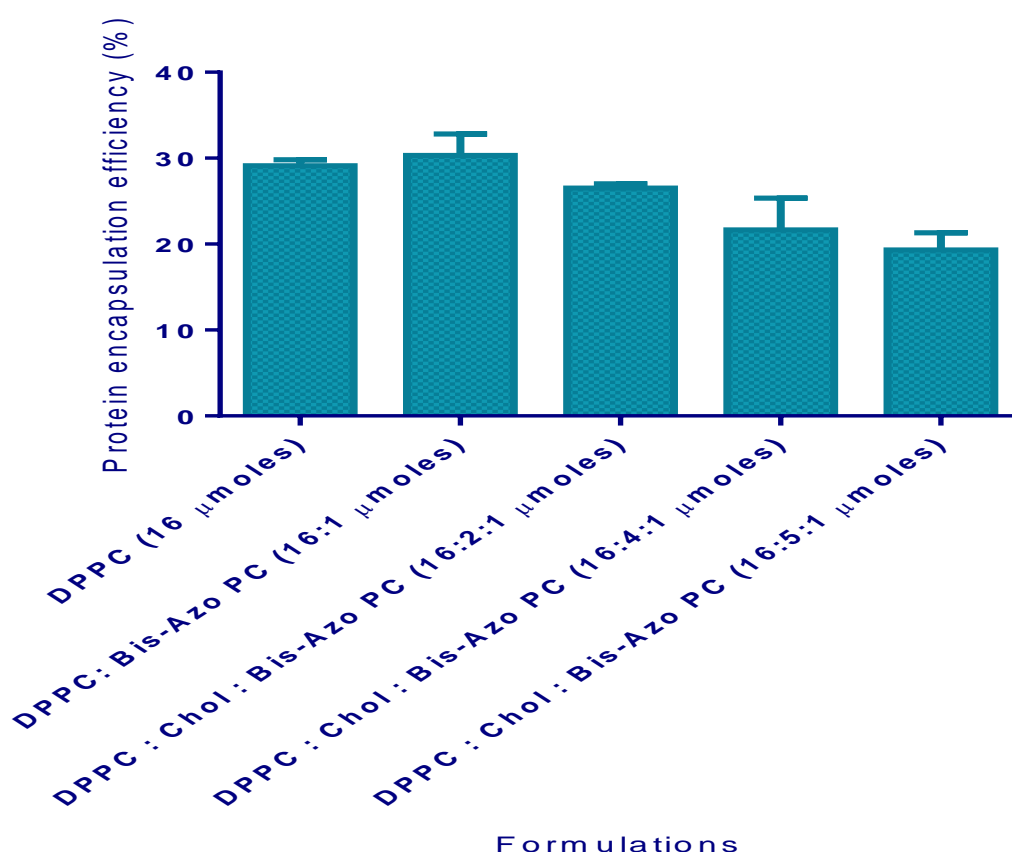


Fig 3.15: Initial protein loading IFV for short-term stability.

IFV were prepared via interdigitation fusion method. IFV composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC, where applicable. Results represent mean \pm SD of n=3 batches.

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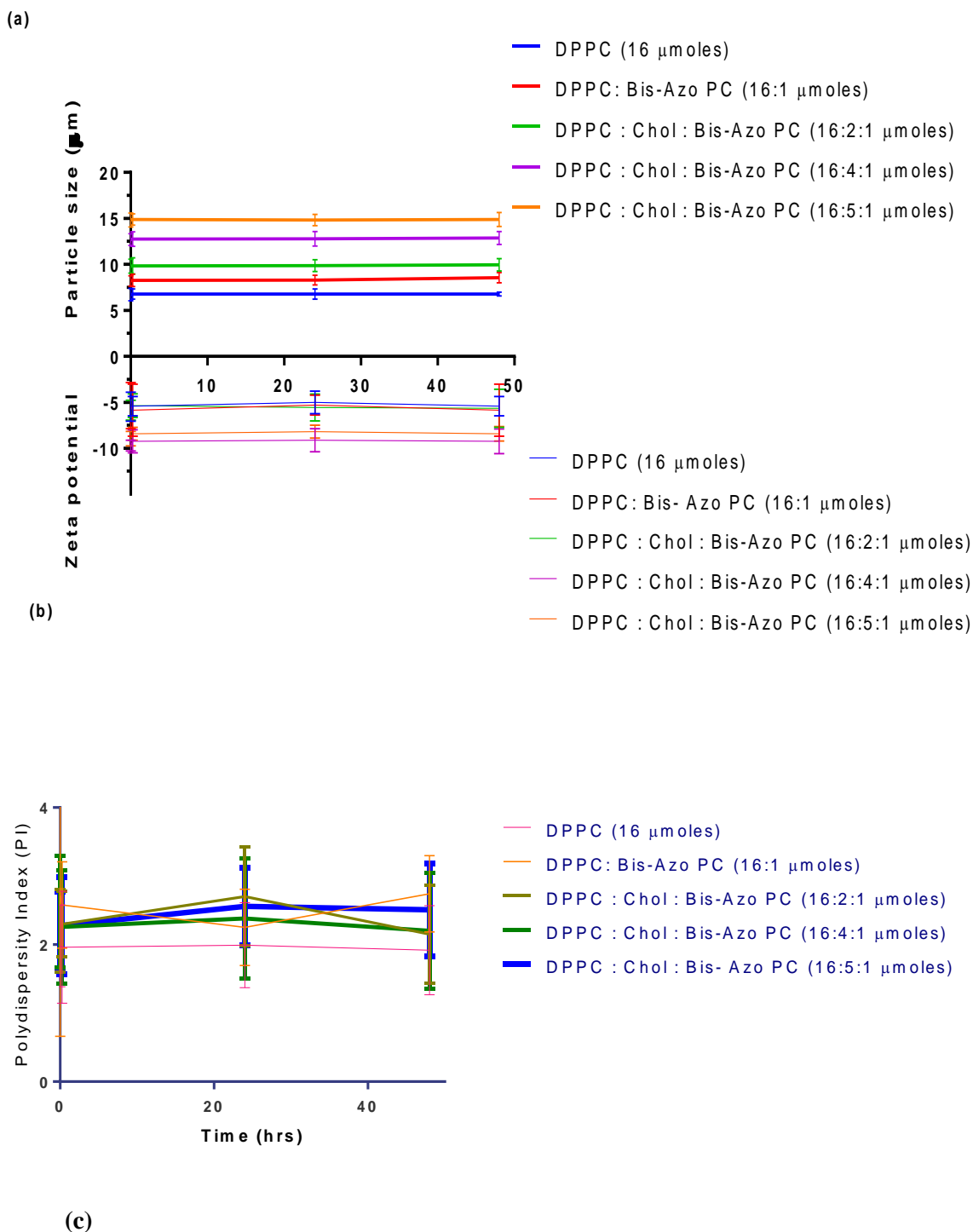


Fig 3.16: Stability assessment of IFV formulations stored at room temp for 15 min, 24h and 48h.

Measurements were taken of (a) Size (µm) and (b) zeta potential (mV) and (c) span measurement after 15 min, 24h and 48h at room temperature. Results represent mean \pm SD of n=3 batches.

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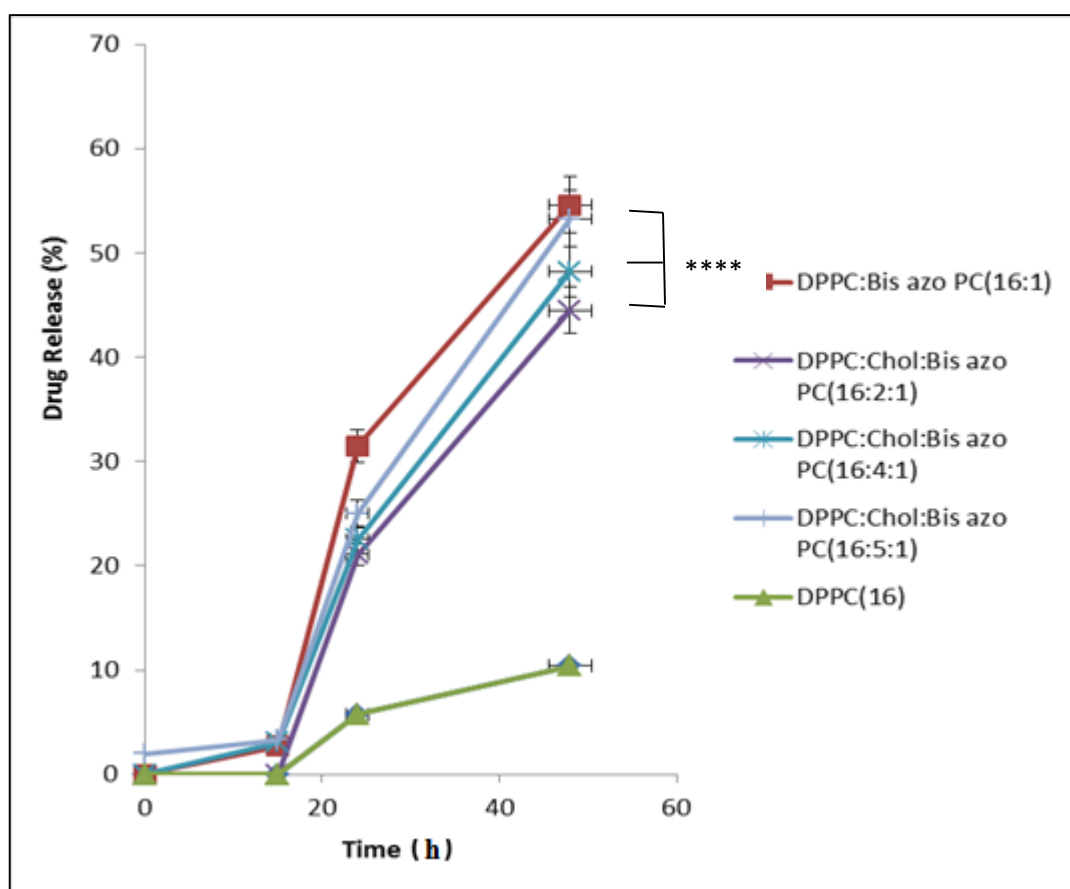


Fig 3.17: Drug leakage (%) from IFV stored at room temp for 15 min, 24 h and 48 h.

IFV: Liposomes were prepared via interdigitation fusion method. Liposomes composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC where applicable. % leakages were taken after 15 min, 24 h and 48 h at room temperature and measured by UV spectrophotometer. Results represent mean \pm SD of n=3 batches. Significance was measured by one-way ANOVA (**** p<0.0001).

Liposomes vesicle size at 25 $^{\circ}$ C has no significant difference when comparing IFV formulations at 15 min, 24 h and 48 h of drug release (Fig 3.16). Although there were some fluctuations over the time points, the general trend suggests IFV were stable over 15 min, 24 h and 48 h. Similarly, there were also no significant changes to the span over the same time period. In terms of zeta potential (Fig 3.16) there was no significant difference in surface charge under storage in the dark at 25 $^{\circ}$ C for two days. This means all IFV were stable in terms of size, size distribution and zeta potential at 25 $^{\circ}$ C for 15 min, 24 h and 48 h. This is because the storage conditions assessed, especially temperature, was not above the transition temperature of the lipid, which is 41 $^{\circ}$ C for DPPC (Mabrey and Sturtevant, 1976). However, according to Casals and co-workers (Casals et al., 2003), neutral liposomes such as DPPC have a tendency to aggregate by Van der Waals force of interaction whereby aggregation phenomenon is more pronounced and common in large vesicles. However, this was not observed in this study (Fig 3.16). The outcome of negative charge indicates the presence of BSA-FITC which acquires negative charge in the PBS at pH 7.4 due to ionisation.

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In terms of liposomal drug retention (Fig 3.17), DPPC IFV 16 μ moles stored in the dark at room temperature exhibited 0 % release. On the other hand, DPPC IFV containing 1 μ moles of Bis-Azo PC exhibited release rates of 2.71 ± 0.15 % (of total amount entrapped) after 15 min. Interestingly, DPPC IFV 16 μ moles stored in the dark at room temperature exhibited release rates of 5.74 % (of total amount entrapped) after 24 h with a total release of 10.42 % following 48 h incubation, whereas DPPC IFV sample containing 1 μ moles of Bis-Azo PC exhibit higher release rates with 31.48 % loss within 24 h and a total of 54.61 % within 48 h when stored in the dark at room temperature. This may be due to the presence of photosensitive lipid, Bis-Azo PC, within DPPC liposomes which causes the photo-isomerisation (trans -cis isomerisation) during the formulation of IFV (Sandhu et al., 1986).

DPPC:Bis-Azo PC IFV containing 2 μ moles stored in the dark at room temperature exhibited 0 % release after 15 min and 21 % and 41 % (of total amount entrapped) after 24 h and 48 h, respectively, suggesting that the percentage release could be reduced with the inclusion of cholesterol in the IFV (Fig 3.17). Furthermore, DPPC:Bis-Azo PC IFV containing 4 μ moles exhibited release rates of 3 % (of total amount entrapped) after 15 min and 22 % (of total amount entrapped) after 24h with a total release of 48 % following 48 h incubation. On the other hand, DPPC:Bis-Azo PC containing 5 μ moles of cholesterol and stored in the dark at room temperature, exhibited higher release rates with 3.3 %, 25% and 53% loss within 15 min, 24 h and 48 h, respectively. It should, however, be noted from Fig 3.17 that IFV prepared using DPPC and DPPC:Bis-Azo PC 16:2:1 shows significantly less leakage of protein as compared to other IFV. Therefore, these were considered stable IFV formulations.

Statistical analyses ($P < 0.05$) shows that the % of leakage from DPPC:Chol:Bis-Azo PC 16:2:1 IFV after 15 min, 24 h and 48 h is statistically significant ($P < 0.0001$) compared to the % of protein leakage from DPPC:Chol:BisAzo PC 16:4:1 and DPPC:Chol:BisAzo PC 16:5:1 (Fig 3.17). There is no significant difference seen between the IFV prepared using DPPC:Bis-Azo PC compared with the IFV prepared using DPPC:Bis-Azo PC 16:2:1. It can thus be said, statistically, that the amount of cholesterol plays an important role in IFV stability. Earlier studies evidenced that the tendency of cholesterol to persuade membrane stability was thought to happen due to the strong interaction between the rigid hydrophobic ring structure of cholesterol molecule and alkyl-side chains of phospholipids that ultimately reduces membrane permeability (Wiseman et al., 1993).

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3.4.6.2. Long- term stability

Subsequent to short-term stability, it was important to assess the long term stability of IFV formulations. Long-term stability study was carried out assessing formulations of DPPC 16 μ moles, DPPC:Bis Azo PC 16:1, DPPC:Chol:Bis Azo PC 16:2:1, DPPC:Chol:Bis Azo PC 16:4:1 and DPPC:Chol:Bis Azo PC 16:5:1. The effect of placing the aqueous dispersion at fridge temperature (at 4 °C) in the dark for two weeks was observed by assessing the three physicochemical parameters of size, size distribution, zeta potential and drug retention inside the liposomes (Figs 3.18, 3.19 and 3.20).

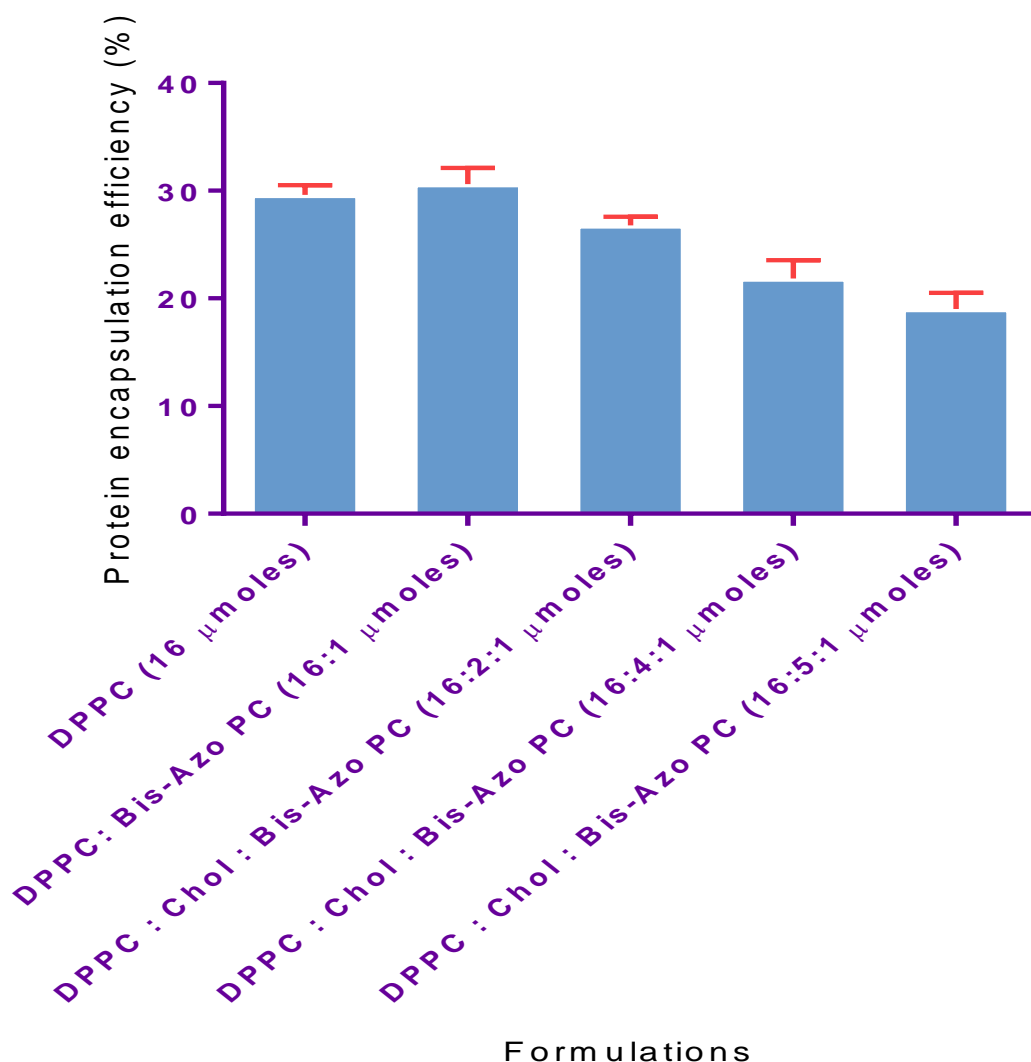
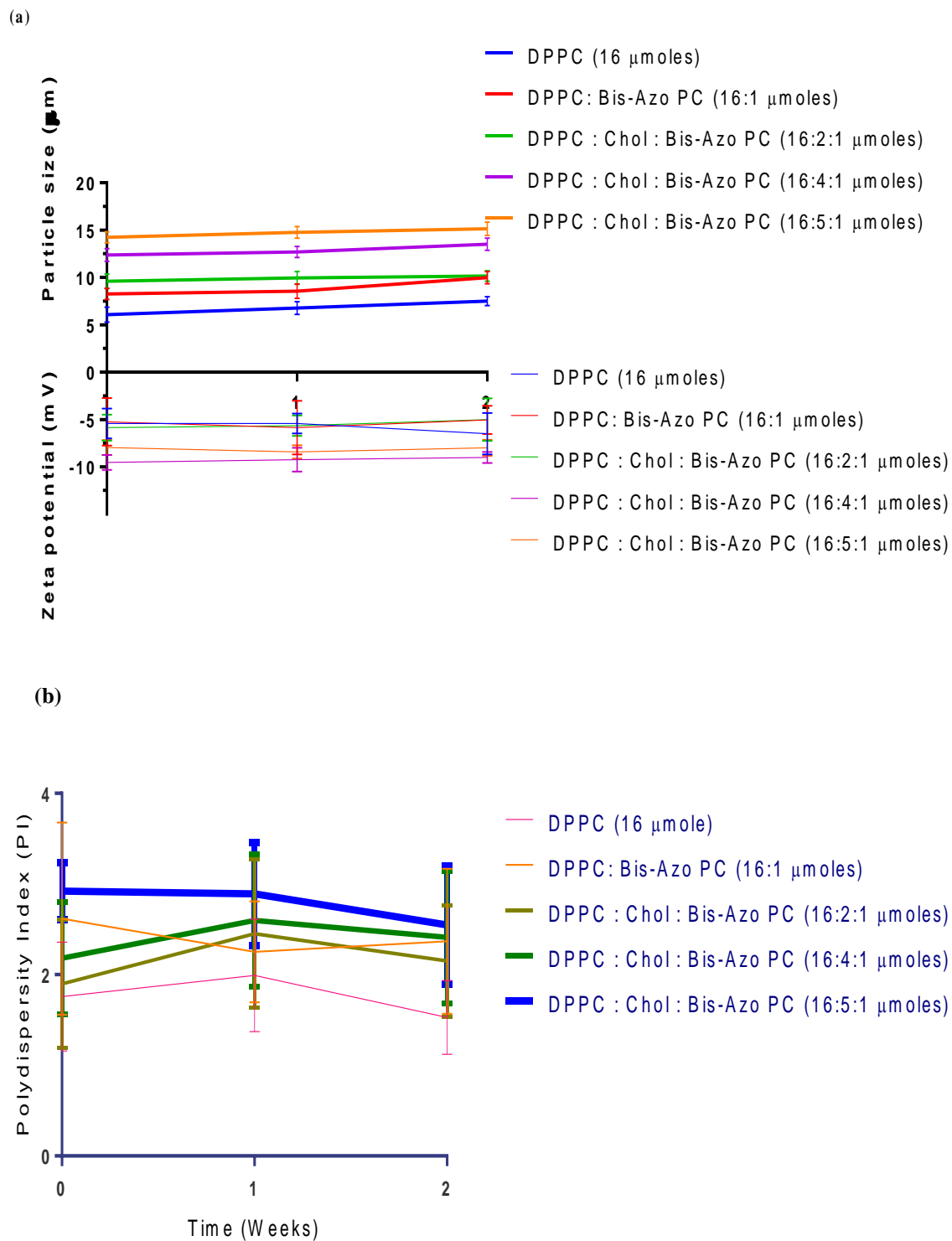


Fig 3.18: Initial protein loading of IFV for long-term stability.

IFV were prepared via interdigitation fusion method. IFV composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC, where applicable. Results represent mean \pm SD of n=3 batches.

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(C)

Fig 3.19: Stability assessment of IFV formulations stored at 4 °C for two weeks.

Measurements were taken of (a) Size (μm) and (b) zeta potential (mV) and (c) span measurement after One week and two week at 4 °C. Results represent mean \pm SD of n=3 batches.

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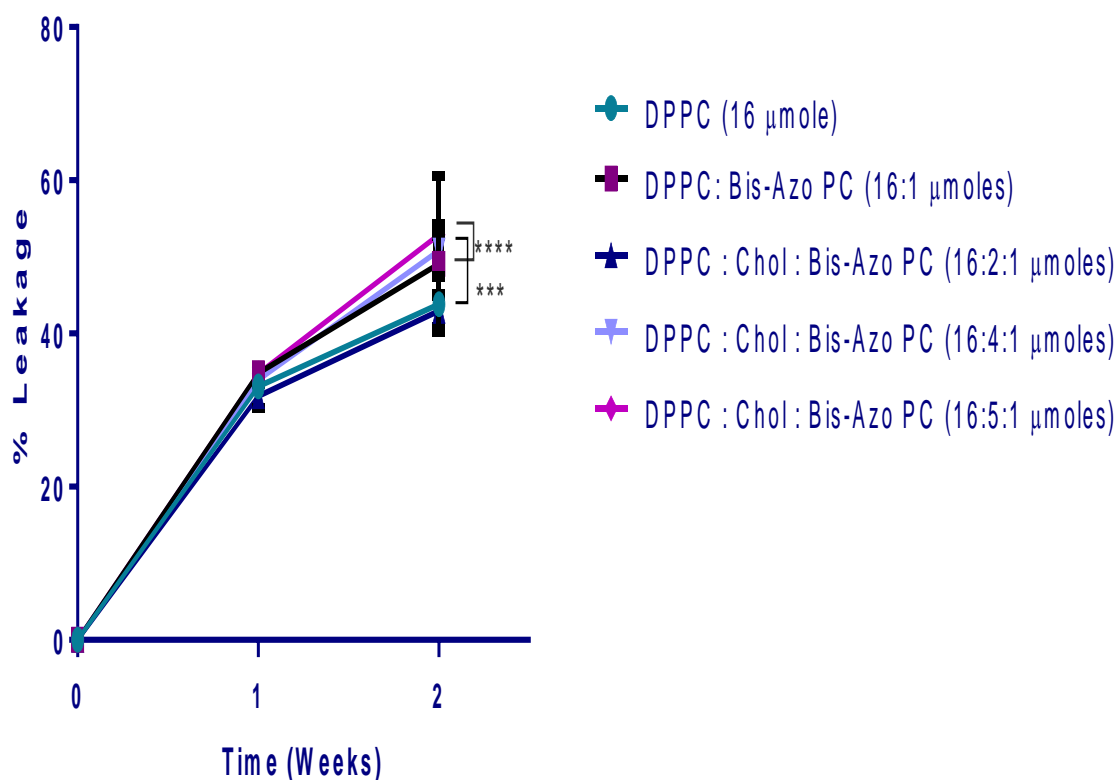


Fig 3.20: % leakage of IFV stored at 4 °C for two weeks:

IFV were prepared by using interdigitation fusion method. Liposomes composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC where applicable Measurements were taken after one week and two week at 4 °C. Results represent mean ± SD of n=3 batches. % leakages were taken after 15 min, 24h and 48h at room temperature and measured by UV spectrophotometer. Results represent mean ± SD of n=3 batches. Significance was measured by one-way ANOVA (**** p<0.0001; *** p<0.001).

It is clear from Fig 3.19 that in terms of size at 4 °C there was no significant changes in size when comparing IFV formulations for two weeks. Nonetheless, there were slight variations in particle size of vesicles that were minor. Similarly, long term stability study has no major influence on the span of IFV (Fig 3.19). In terms of Zeta potential measurement (Fig 3.19), there was no significant difference in surface charge of IFV comparing one week and two weeks of storage in the dark at 4 °C. This result suggests that IFV are stable at 4 °C for two weeks. The outcome of negative charge was due to the BSA-FITC, otherwise DPPC IFV without BSA-FITC shows neutral charge. It is worth noting that the enhanced physical stability of BSA-FITC stored at 4 °C in refrigerator confirms the interpretation of (Plessis et al., 1996) who demonstrated that good stability in terms of size, span and zeta potential should be achieved by storing liposomes in fridge at 4 °C.

As can be seen from Fig 3.20, DPPC interdigitation fusion vesicles (IFV) which are stored at fridge temperature exhibit a release rate of 33.11 % (of total amount entrapped) after one week and a release rate of 43.86 % following two weeks of storage. On the other hand, DPPC IFV sample

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containing 1 μ moles of Bis-Azo PC shows higher release rates, with 34.86 % of the total entrapped amount lost within one week and 49.10 % within two weeks following storage at the same temperature. This further demonstrates that the inclusion of Bis-Azo PC within liposomes influences the % leakage of protein. In comparison to short-term stability, % release differences between these IFV were significantly ($P < 0.05$) low owing to the storage condition at 4 $^{\circ}$ C.

It is also observed from Fig. 3.20 that the extent of the leakage was reduced in DPPC: Cholesterol: Bis-Azo PC (16:2:1), with 31 %, 42 % release after one week and two weeks, respectively. This further cements the hypothesis that cholesterol plays a key role in liposomal stability. Previous studies clearly indicate that the amount of cholesterol, alters the fluidity of liposomes by modifying the orientation of mobile hydrocarbon chains of liquid crystalline phospholipid bilayer which reduces the permeability behaviour and increases the vesicle drug retention mechanism; in theory it diminishes gel-to-liquid phase transition of lipid bilayer (Vilcheze et al., 1996; Ohtake et al., 2005; Vingerhoeds et al., 1994). Interestingly, IFV prepared using the supplementation of DPPC:Bis-Azo PC with 4 and 5 μ moles of cholesterol results in higher release rates of 33.98 % and 34.78 % (of total amount entrapped) respectively after one weeks; with a release of 50.86 % and 52.9 % respectively following two weeks of storage in the dark at 4 $^{\circ}$ C. This suggests that as the amount of cholesterol increases the IFV stability decreases (Fig. 3.20).

Statistical analyses ($P < 0.05$) show that the difference in protein leakage from IFV DPPC:Chol:Bis-Azo PC (16:2:1) vs protein leakage from IFV DPPC:Chol:Bis-Azo PC (16:4:1) was statistically significant ($P < 0.001$) while % of protein leakage from IFV DPPC:Chol:Bis-Azo PC (16:2:1) vs % of protein leakage from IFV DPPC:Chol:Bis-Azo PC (16:5:1) was very significant ($P < 0.0001$) and % of protein leakage from IFV DPPC:Chol:Bis-Azo PC (16:2:1) vs % of protein leakage from IFV DPPC:Bis-Azo PC (16:1) was very significant ($P < 0.0001$) (Fig 3.20). All the protein release data in Fig 3.20 passed the normality test using one-way analysis of variance (ANOVA) and a multiple comparison post-test Tukey.

Overall, short-term and long-term stability studies show that the DPPC: Chol: Bis-Azo PC (16:2:1) should be the better choice for drug release as compared to other IFV. However, long- term shelf life of these IFV should be improved by the use of freeze-drying (lyophilisation) technique (Mohammed et al., 2004b) in which water gets removed from the liposomes and thus liposomes are stored as freeze-dried powder form.

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3.5. Conclusion

In conclusion, IFV with or without Bis-Azo PC sensitized were successfully prepared by ethanol induced fusion method. This technique is beneficial and eye-catching because it produces qualitative and similar vesicles which are confirmed through confocal microscopy and IFV characterisation methods, such as, size analysis.

Within this study, the one of aim of this research was to quantitatively and qualitatively estimate the impact of cholesterol content on the stability of IFV prepared. Results revealed that cholesterol has a major impact on the stability of IFV prepared. These findings from this study mirror conclusions from previous researchers (Mohammed et al., 2004b) who found that the presence of cholesterol improves bilayer stability, and that, in spite of bilayer charge, liposomes rich in cholesterol were more stable than cholesterol free liposomes.

Formation of SUV from MLV is a very important step in IFV formation. For that reason we prepared SUV by bath and probe sonication. It can be concluded based on the results of this study that SUV formation using both types of sonication depends on the liposome composition and cholesterol content. Preliminary results validate the suggestion that IFV without cholesterol forms SUV within a short time whereas IFV with cholesterol takes more time for SUV formation. Interestingly, insertion of enhanced cholesterol increases the sonication time and influences SUV formation.

Our results also demonstrated that incorporation of protein into liposomes was seen to be influenced by the IFV cholesterol. Enhanced cholesterol incorporation in neutral liposomes i.e. DPPC liposomes, was shown to coincide with an increased vesicle mean volume diameter; although the presence of cholesterol did not significantly influence the zeta potential of any of the IFV formulations tested. It is also apparent from the studies that the amount of cholesterol also influences the encapsulation efficiency of protein.

Photo-triggered release study showed that rapid and controlled release of protein in a programmed fashion was achieved by using Bis-Azo PC sensitized IFV. However, the release of entrapped protein is again dependent on the amount of cholesterol and lipid composition. Therefore, IFV prepared using DPPC:Chol:Bis-Azo PC (16:2:1) and DPPC:Chol:Bis-Azo PC (16:4:1) could give better release as compared to other IFV. But IFV prepared using DPPC:Chol:Bis-Azo PC (16:4:1) have less entrapment efficiency than the IFV prepared using DPPC:Chol:Bis-Azo PC (16:2:1). These findings also demonstrate that based on the power and intensity of UV lamps, photosensitive IFV give different patterns of release of entrapped protein. Indeed, ROHS 36 w professional UV curing lamp was superior to the other two UV lamps, cognizant of the expected release pattern and thus was used for further investigations. Photo-induced release study also points out that 100 %

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release is not achievable. Nevertheless, DPPC:Chol:Bis-Azo PC IFV (16:2:1) could give 84 % release after 12 min UV irradiation.

Stability studies showed that inclusion of increasing amounts of cholesterol, decreases the stability of IFV and DPPC:Chol:Bis-Azo PC IFV (16:2:1) could be better choice in comparison to other IFV formulations. It is also revealed from stability studies that IFV formulations remain stable at 4 °C for two weeks and no significant leakage is seen.

Confocal microscopy study evidences that IFV are bigger than SUV and smaller than MLV. Moreover, confocal study established that BSA-FITC was successfully incorporated into MLV, SUV and IFV formulations.

Overall, photosensitive IFV prepared using DPPC:Chol:Bis-Azo PC (16:2:1) seems to be the optimal formulation for controlled drug release under UV light. This IFV could have practical applications in areas, such as photo-stimulated drug delivery. Even though UV light does not penetrate deeply into the tissues, these types of photosensitive IFV would be useful in photodynamic therapy in accessible areas such as skin, eyes and mucous membrane. They would also be beneficial in cosmetic industry, especially in “sun-tanning apparatus” and ocular drug delivery.

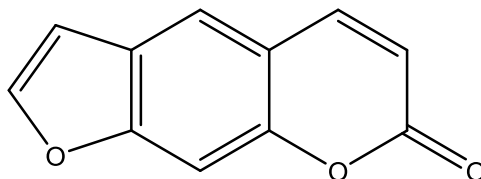
CHAPTER 4

Development of Photosensitive Liposomes for the Controlled Release of 4'-(6- Hydroxyhexyloxy) methyl-4, 5', 8- trimethylpsoralen (HMT)

Chapter 4- Development of Photosensitive Liposomes for the Controlled Release of 4'-(6-Hydroxyhexyloxy) methyl-4, 5', 8-trimethylpsoralen (HMT)

4.1. Introduction

Psoralen (Fig 4.1), also known as psoralene, belongs to the family of furocoumarin and is derived from the fusion of furan ring with a coumarin that is found naturally in a few plant species such as in the seeds of *Psoralea corylifolia* and fruits of many plants, such as, fig, lemons, celery, parsley and limes, or is synthesized *in vitro* (Charles and Evans, 2002).



7H-furo [3, 2-g] chromen-7-one

Fig 4.1: Psoralen compound.

Psoralen has shown a broad spectrum of biological activities which include photosensitization, cytotoxicity, phytotoxicity, insecticide, antibacterial, antifungal (Bourgaud et al., 2006; Chen et al., 2012; Gambari et al., 2007; Ishikawa et al., 2009). Moreover, psoralen has been recommended as a potential therapy for the treatment of cancers (Francisco et al., 2012; Kawase et al., 2005; Scaffidi et al., 2011), autoimmune diseases (Strauss et al., 2000) and skin disorders, such as vitiligo and psoriasis (Adişen et al., 2008; Grimes, 1997).

Much research in recent years has focused on use of psoralen in Psoralen Ultra-Violet A (PUVA) treatment for psoriasis. Psoriasis is T-cell mediated multifactorial type-1 autoimmune disorder in which the human immune system mistakes a normal skin cell for a pathogen, due to genetic and environmental factors, and sends out defective signals that cause overproduction of new skin cells (Voorhees, 1996). It generally occurs at any point in the lifespan, affecting children, teenagers, adults and older people. As per the survey of US National Institute of Health, 2.7 % of the world population suffer from psoriasis. A few researchers have proven that psoriasis patient suffers from depression, cardiovascular disease and an impaired quality of life (Nijsten and Wakkee, 2009). It also shows different symptoms: skin lesions, cracking of skin, inflammation, itching, joints pain and scaly dots on the skin. Various therapies, such as topical therapies and phototherapy can be applied to treat psoriasis (Griffiths et al., 2000). If psoriasis is not controlled by topical treatments, phototherapy or light therapy maybe recommended. Phototherapy involves the irradiation of human skin with UV light on a regular basis under medical supervision (Gawkrödger, 1997). There are

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two types of ultraviolet (UV) light which can be used for the treatment: ultraviolet A (UVA) (315-380nm) and ultraviolet B (UVB) (280-315nm). They are different parts of normal sunlight. Due to the fact that human skin is sensitive to UVB, psoriasis can be readily treated with UVB exposure alone. In contrast, UVA directly penetrates the body but requires a sensitizing medication, such as psoralen to treat psoriasis. This process is known as PUVA therapy (psoralen combined with ultraviolet A treatment), which is currently approved by the US Food and Drug Agency for clinical applications (Gelfand, 2007; Stern, 2007).

There are several routes through which psoralen can be administered as part of PUVA therapy (Griffiths et al., 2000). Firstly, psoralen in a capsule dosage form can be taken orally two hours prior to the treatment with UVA light. Alternatively psoralen can be added to bath water, known as bath PUVA or psoralen can be administered as a topical application of psoralen gel or cream to small areas of psoriasis about half an hour prior to UVA light treatment.

PUVA therapy is conducted using one of three psoralen derivatives, i.e. 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and 4, 5, 8-trimethylpsoralen (TMP). All these psoralens possess planar aromatic structure and UV absorption bands in the range of 200-350 nm. Because of planar aromatic structure, psoralens can easily intercalate with DNA bases. Upon irradiation with light of proper wavelength, a psoralen molecule absorbs photons in the ground state and shifts an electron to the excited mode (Bryantseva et al., 2008). Subsequently, the adducts with thymine and cytosine can be produced that would lead to slower cell proliferation (Fig 4.2). This process may also increase tyrosinase activity and encourage the synthesis of melanocytes adjacent to the injured cells.

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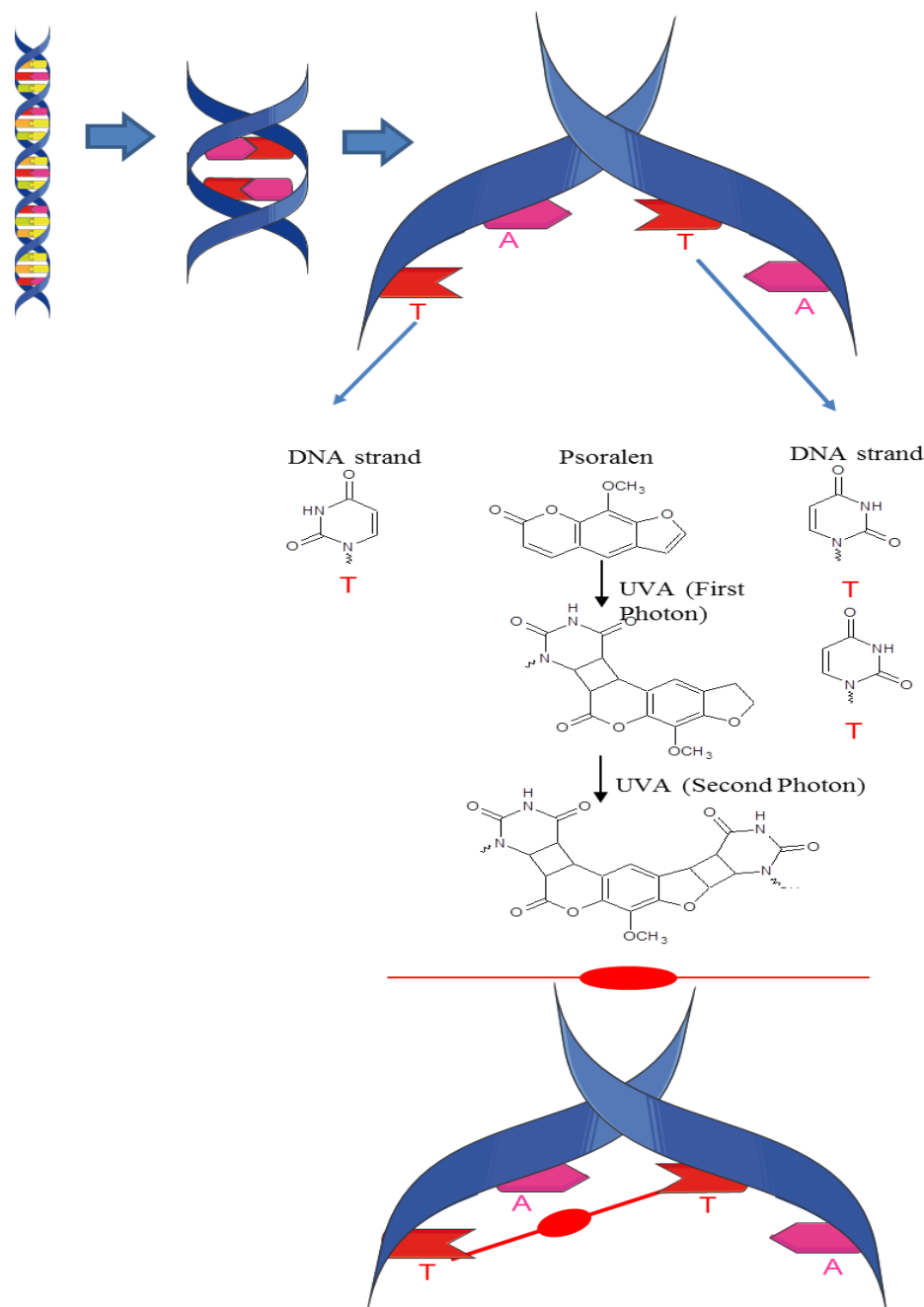


Fig 4.2: Interstrand crosslinking between psoralen and DNA base pairs.

Cells get damaged when a psoralen is cross-linked into a nucleic acid duplex in sites containing two nucleic acid base pairs (either thymines or uracils) on opposing strands sequentially absorbing 2 UVA photons. Unfortunately, topical delivery of psoralen in psoriatic skin faces a number of challenges. Firstly, as pointed out by Morganti and co-workers (Morganti et al., 2001), stratum corneum (SC) acts as an “active wall” that opposes the penetration of xenobiotic i.e. a foreign

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chemical substance, such as psoralen, and it is also apparent that the major route of penetration across the SC is intercellular lipids (Hadgraft, 1996). Secondly, during psoriasis, psoriatic skin turns rigid due to increment in the level of cholesterol and reduction in the level of ceramides (Wertz et al., 1989). Lastly, due to “rigidization”, psoriatic skin becomes deficient in normal moisturizing factors (NMFs), such as water. Thus, targeting psoriatic tissue via topical delivery of psoralen is very difficult. However, this problem can be solved by the use of liposomes, the lipoidal carrier systems. Liposomes unravel the problem of lipid imbalance by providing the linoleic acid that does repair the normal skin conditions (Morganti et al., 2001). Moreover, liposomes are non-irritant and can be easily excreted through the body. Indeed, it has been proven that liposomes are effective drug carrier systems across the psoriatic skin (Menter et al., 2009). Therefore, in this study, photosensitive LUV was used to entrap a psoralen derivative. In order to increase drug entrapment efficiency of photosensitive LUV, LUV-entrapped psoralen were prepared using two common techniques: interdigitation fusion method (as described in Chapter 3) and extrusion method.

Large unilamellar vesicles by extrusion method (LUVETs) is a technique in which micrometric liposomes, such as MLV suspensions, are forced through polycarbonate filters (100 nm of pore size) to generate LUV possessing diameters near the pore size of the polycarbonate filters used (Hope et al., 1985; Olson et al., 1979). Preceding extrusion through the polycarbonate filters, MLV suspensions are generally disrupted either via several freeze-thaw cycles or via prefiltering of the MLV suspensions. This process thwarts the lipid membranes from fouling and recovers particle size distribution homogeneity of the final suspension. Moreover, the extrusion should be carried out at temperatures above the T_c of the phospholipid because below the T_c , the lipid a vesicle has a tendency to entwine with rigid membranes that cannot permit via the pores (Mozafari et al., 2008; Nayar et al., 1989). Several studies on the size of the extruded liposomes as a function of concentration and pressure has established that the size of the liposomes reduces slightly as the extrusion pressure is elevated (Kolchens et al., 1993). The advantages of using the LUVETs are that this method can be useful for a wide variety of lipid species and mixtures; it works directly from MLV formulations, and is very rapid compared to other methods (Hope, 1993; Patty and Frisken, 2003). The extrusion method can generate lipid vesicles with precisely controlled sizes, though this technique is limited due to the requirement of highly specialized equipment (Pradhan et al., 2008). Recently, a few researchers (Morton et al., 2012) have validated that extrusion, through constant pressure-controlled extrusion apparatus, does not influence the entrapped drug because drug entrapment is accomplished before the extrusion and that liposomes do not lose their biophysical stability. In the present study, extrusion through Avanti mini extruder was performed. Furthermore, standard polycarbonate nanomembranes with a pore size of 1 μm were employed in this method, which allowed a predetermination of desired liposomes. According to Janoff and co-

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workers (Janoff et al., 1991), the polydispersity of liposomes diminished with the increasing number of extrusion cycles. Thus, in the present study, liposomes were extruded through the polycarbonate membranes for several times in order to minimise the polydispersity of the prepared liposomes.

Numerous experiments have demonstrated that psoralen solubility can influence the intercalation between the psoralen and DNA duplexes (Hearst et al., 1978). The same research group also revealed that the interstrand crosslinking occurs only when psoralen molecules are present in the right position and at the right time. The availability of psoralen molecule at the proper place is related to the solubility of psoralen in aqueous solution and upon the dissociation constant resulting from non-covalent binding of psoralen with DNA. Hence, the higher the solubility, the greater the number of psoralen molecules available for intercalation and ultimately psoralen solubility is very important for psoriasis treatment. For this reason, psoralen derivative i.e. 4'-(6-Hydroxyhexyloxy) methyl-4, 5', 8-trimethylpsoralen (HMT) was used in this project, as it has high aqueous solubility and fast photochemical kinetics compared to other psoralen derivatives (Isaacs et al., 1982). The higher solubility of HMT is due to presence of 6-Hydroxyhexyloxy i.e. OH containing side chain. Apart from this, HMT is well known for its use in the study of secondary structure of DNA (Piette and Hearst, 1983).

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4.2. Aims and objective

As discussed in the previous section (4.1), it has been proven that psoralen along with 320-400 nm UVA light is an effective treatment for a variety of dermatologic disorders such as psoriasis and vitiligo. Unfortunately, topical delivery of psoralen in psoriatic skin faces a number of challenges, however it has been reported that liposomes are effective drug carrier systems across the psoriatic skin (Menter et al., 2009). Furthermore, it has been demonstrated that a higher psoralen dose has a greater success rate than a lower dose and needs a lower mean cumulative UV dose to achieve success (Mouli et al., 2013). The aim of this study is to look at whether it is possible to achieve a higher psoralen dose through a photo-activated burst release of psoralen i.e. to apply psoralen gel containing photo-active liposomes to the areas of psoriasis to achieve the release of psoralen and treating the affected areas with UV irradiation.

The objectives of this work were:

- Incorporation of Bis-Azo PC (Chapter 2) into a mixture of DPPC and cholesterol to a ratio of 16:2:1 (DPPC: Chol: Bis-Azo PC), to form IFV and to entrap HMT.
- Morphological analysis of HMT-entrapped IFV and empty IFV through confocal microscopy
- Use of ROHS 36w professional UV curing lamp for the photo-triggered release.
- Quantification of the amount of HMT release from IFV using UV spectroscopy.
- Determination of the liposomal stability in order to detect the leakage of HMT using UV spectroscopy.

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4.3. Materials and Methods

4.3.1. Materials

4.3.1.1. Chemicals

Chloroform and Methanol were from Fisher Scientific, Loughborough, UK. Triton- X; and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich, Poole, and Dorset, UK. Distilled water was used throughout. All the chemicals and reagents used were of analytical grade.

4'-(6-Hydroxyhexyloxy) methyl-4, 5', 8-trimethylpsoralen (HMT), with chemical structure shown in Fig.4.3 was purchased from Berry & Associates, Inc., USA.

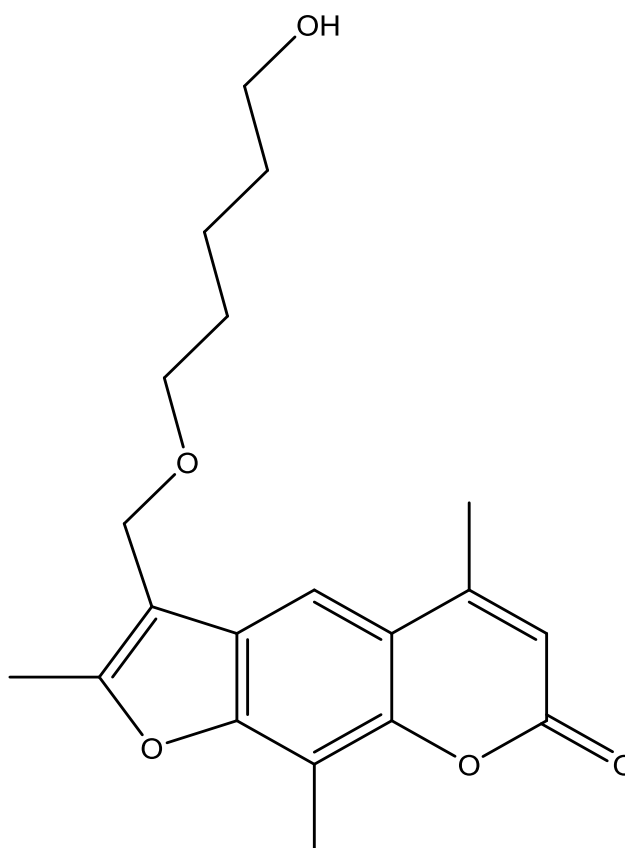


Fig 4.3: Chemical structure of HMT.

4.3.1.2. Lipids

1, 2-Dimyristoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (its chemical structure can be shown in section 3.3.1.2.) were purchased from Avanti Polar lipids, Alabama, USA.

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4.3.2. Methods

4.3.2.1. Synthesis of photosensitive lipid Bis-Azo PC

The chemical synthesis of photosensitive lipid 1, 2-bis (4-(n-butyl) phenylazo-4'-phenylbutyroyl) phosphatidylcholine (Bis-Azo PC) was as per the previously described method by Sandhu and co-workers (Sandhu et al., 1986) which is described in chapter 2 (Section 2.3.3.4).

4.3.2.2. Preparation of LUV

Photosensitive LUV were prepared using two methods: a) Interdigitation fusion method and b) Extrusion method.

4.3.2.2.1. Preparation of photosensitive IFV by interdigitation fusion method

Photosensitive IFV were prepared as described by Ahl and co-workers (1994) (Ahl et al., 1994). In brief, MLV were prepared by thin film hydration method using DPPC, cholesterol and Bis-Azo PC in ratio of 16:2:1. The lipids were dissolved in a mixture of chloroform and methanol (9:1, v/v) at a concentration of 10 mg/mL. The solvent was then evaporated under reduced pressure using rotary evaporator (Buchirotavapor-R 3) at 150 rpm for 15 min. Intermittent vortexing at 55 °C resulted in deposition of thin dry lipid film on the sides of the flask which was further dried with a stream of nitrogen to remove any residual solvent. The lipid film was then hydrated with 2 mL of a 10 µg/mL hydration media (PBS) and then mixed, at intermittent vortexing, with 4'-hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) which had been prewarmed above the transition temperature [T_m (41 ± 1 °C)] of the lipids. The mixed suspension was heated at 50 °C for 30 min until a milky coloured suspension was obtained. The resulting multilamellar vesicles were sonicated using a bath sonicator (Fischer Scientific, UK) for 35 min at 55 °C. Moreover, absolute ethanol was added to the SUV suspension while stirring at room temperature until the ethanol concentration in the sample was 4 M. The samples were then instantly vortexed. This process quickly altered the transparent SUV suspension into an extremely viscous, opaque white suspension of interdigitated phospholipid sheets. Following the inclusion of absolute ethanol, the samples were sealed and incubated for 15 min at room temperature, then incubated for 15 min at 50 °C ($> T_m$ of the lipid). The caps of the sample vials were then loosened to allow ethanol evaporation, and the incubation was continued for another 30 min at the same temperature. Following this, the samples were bubbled at a temperature of above the T_m with a gentle stream of N_2 to remove ethanol. Subsequently, these samples were washed three times with a solution containing 50 mM NaCl, 10 mM Tris-Hcl (pH 7.4) by centrifugations for 30 min at 27,000×g at 4 °C using Beckman coulter ultracentrifuge equipped with TLN-100 rotor until no drug could be detected in the supernatant.

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The resultant pellets were then suspended with PBS and the vesicles stored at 4 °C. Similarly, photosensitive IFV (DPPC:Chol:Bis-Azo PC 16:2:1) without HMT were prepared via interdigitation fusion method using 2 mL of PBS in the place of HMT in PBS .

4.3.2.2.2. Preparation of photosensitive IFV by extrusion method

This was carried out by following the procedures below

1. Photosensitive multilamellar vesicles were prepared by vortexing dry lipid (DPPC:Chol:Bis-Azo PC16:2:1 μ moles) in the presence of a prewarmed solution of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (2 mL, 10 μ g/mL) in PBS.
2. In order to increase the entrapment efficiency of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen, photosensitive multilamellar vesicles suspension were subjected to 1 to 3-5 freeze/thaw cycles by alternately placing the sample vial in a dry ice bath and warm water bath.
3. Photosensitive LUV were produced by transferring the resulting photosensitive multilamellar vesicle dispersion into a mini extruder (manufactured by Avanti Polar Lipids, INC., Fig 4.4), which allowed the extrusion of the multilamellar vesicles through standard 19 mm polycarbonate filters with 1.0 μ m pore size.
4. Similarly, photosensitive LUV (DPPC:Chol:Bis-Azo PC 16:2:1) without HMT were prepared via extrusion method using 2 mL of PBS.

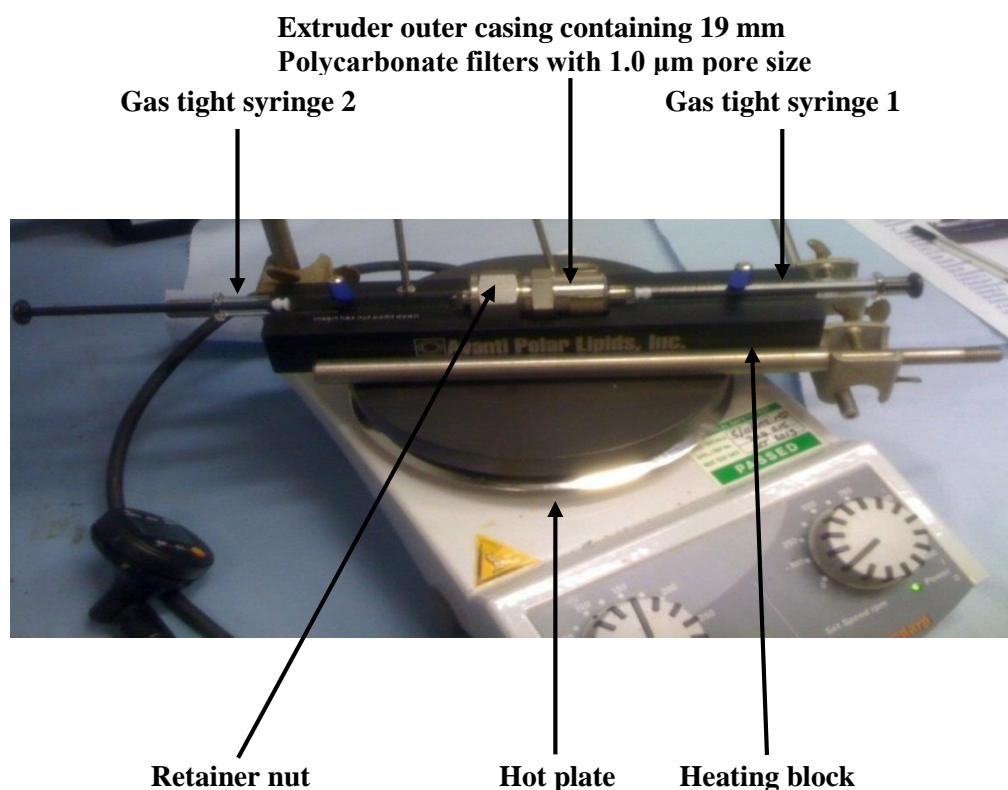


Fig 4.4: Lipid mini extruder for LUV preparation.

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4.3.2.3. Liposome characterisation

4.3.2.3.1. Particle size measurement

The average diameter and polydispersity index (P.I.) of the MLV and LUV were determined by Dynamic light scattering (DLS) using a Sympatec (Helos, UK) particle sizer. For the measurement of size, 100 μ L of a liposome formulation was pipetted into a glass quartz cuvette (with a marked signal of at least 15 % was being mandatory for an ample reading) containing distilled water and stirred at 1200 rpm to reduce inter-particulate aggregation, and thereafter, the laser beam was focused using Helos software, and the assay was repeated in triplicate.

For SUV, the average diameter and polydispersity index (PDI) were determined by using a Zeta plus (Brookhaven Instruments, UK). Concisely, 100 μ L of SUV suspension was diluted to 4.5 mL with double-distilled water and the z-average diameter of SUV was recorded at 25 $^{\circ}$ C. Measurements were reported as a mean value of three readings and each reading was an average value of measurements recorded for 3 min. The P.I. was used as a measure of the width of the size distribution.

4.3.2.3.2. Zeta potential measurement

Zeta potential was measured using the Zetaplus (Brookhaven Instruments, UK) by means of the photon correlation spectroscopy, which measures the particle electrophoretic mobility in a thermostated cell. For the zeta potential measurement, 100 μ L of liposomal dispersions were diluted to 4.5 mL with 0.01 M PBS prior to analysis and the final measurements were the mean values of ten readings.

4.3.2.4. 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) standard curve

Using a Varian Cary 1 spectrophotometer, the λ_{max} of HMT was determined by measuring its UV spectrum and its λ_{max} was found to be at 249 nm. A stock- solution of HMT (1 mg/mL) was prepared by dissolving 1 mg of 4'-hydroxymethyl-4, 5'-8- trimethylpsoralen (HMT) in 1 mL of phosphate buffer saline solution at pH 6.8. A set of standard solutions of trimethylpsoralen in the range of 0.0786 to 20 μ g/mL were prepared by dilution of the above stock solution with PBS buffer. UV absorbances of all these solutions were measured at 249 nm with 0.01 M PBS (pH 7.4) as the reference. A calibration curve was constructed by plotting absorbance versus concentration of the above standard solutions (path length was fixed at 1 cm). Results of regression analyses and the correlation coefficients (R^2) are demonstrated in Appendix-2.

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4.3.2.5. Determination of HMT entrapment efficiency in IFV

The loading efficiency of 4'-hydroxymethyl-4,5,8-trimethylpsoralen (HMT)-entrapped interdigitation fusion vesicles was determined by measuring the supernatant of IFV present in 3.9 mL wash media (PBS, 0.01M) following separation of IFV by centrifugation in TLN-100 rotor of a Beckman coulter ultracentrifuge for 30 min at 27,000 rpm ($\times g$) at 4 °C. This washing stage was repeated a second time in order to make sure that free HMT are separated from IFV (Casals et al., 1996). The amount of HMT in supernatant was analysed using UV spectroscopy (Varian Cary 1 spectrophotometer) at 249 nm. The entrapment efficiency (EE %) values expressed as entrapment percentage for the prepared IFV were then calculated according to the following equation (Ghanbarzadeh and Arami, 2013; Hathout et al., 2007):

Drug content = Initial amount of drug added – drug remaining in supernatant after centrifugation

$$\text{EE \%} = (\text{drug content} / \text{total amount of drug added initially}) \times 100 \dots \text{Equation (4.1)}$$

4.3.2.6. Fourier transform infrared spectrum (FTIR) study

Fourier transform infrared spectroscopy analysis was performed on a Nicolet iS5 FT-IR spectrometer using a Miracle Micro ATB attachment with diamond crystal (Thermo scientific, UK). Subsequent to the formulation steps, photosensitive IFV with or without HMT were centrifuged at the speed of 27,000 rpm for 30 min at 4 °C using Beckman coulter ultracentrifuge equipped with TLN-100 rotor and supernatant was removed. The particle sediments were subjected to freeze drying overnight. After that, small amount of IFV powder samples were placed on the diamond crystal and compressed gently using the pressure clamp. The diamond crystal was cleaned carefully with 95 % ethanol prior to each use. Measurement was performed in transmission mode in the range of 4000-400 cm^{-1} region with a resolution of 4 per cm^{-1} from 64 parallel scans. The obtained spectra were documented and evaluated using Omnic software version 4.1b (Thermo scientific) software. Similarly, HMT powder samples were analysed and spectra were recorded.

4.3.2.7. Photo-triggered release of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen-entrapped interdigitation fusion vesicles

With the desire of 100% HMT release from liposomes, IFV (DPPC:Chol:Bis-Azo PC (16:2:1) formulations with and without 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen that had been stored in the dark were taken and irradiated using a ROHS 36 w professional UV curing lamp for different time intervals i.e. 0, 1, 3, 6, 9, 12 and 15 min respectively. The samples were then diluted, centrifuged and the amount of HMT in the supernatant was determined HMT using UV assay as described above in sections 4.3.2.4 and 4.3.2.5.

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4.3.2.8. Determination of the short-term stability of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT)-entrapped interdigitation fusion vesicles

In order to evaluate the short term stability of the formulated IFV during storage, liposomes were stored at room temperature (~23 °C) in the dark and evaluated at the following time courses: 3, 6, 9, 12, 15 min, 24 h, 48 h and 96 h; both physical and chemical stability of liposomal formulations were evaluated. Physical stability of IFV was assessed by visual observation for particle size determination after specified time intervals (i.e. 3, 6, 9, 12, 15 min, 24 h, 48 h and 96 h) and chemical stability of IFV was determined by calculating the percentage leakage of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen from the IFV after specified time intervals (i.e. 3, 6, 9, 12, 15 min, 24 h, 48 h and 96 h) through the UV assay as described in sections 4.3.2.4 and 4.3.2.5.

4.3.2.9. Determination of the long-term stability of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT)-entrapped interdigitation fusion vesicles

In order to estimate long term stability, both physical and chemical stability of IFV, the formulated IFV were stored at fridge temperature (~4 °C) in the dark for one and two weeks. Physical stability of IFV was assessed by visual observation for particle size determination after one and two weeks of sample storage while chemical stability of IFV was analysed by checking the percentage leakage of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen from the IFV after one week and two weeks of sample storage, using the UV assay method, as described in sections 4.3.2.4 and 4.3.2.5.

4.3.2.10. Confocal microscopy

Leica confocal microscope was used and IFV prepared using interdigitation fusion method and extrusion methods were examined under a 60 X oil objective along with Helium and Argon lasers. Following the formulation steps, the un-entrapped HMT was removed through centrifugation for 30 min at 27,000×g at 4 °C using Beckman ultracentrifuge and subsequently, liposomal formulation was placed into a microslide and a cover slip was mounted on it to avoid immediate drying out of sample. Furthermore, confocal microscopy was used for the HMT analysis.

4.3.2.11. Statistical analysis

Graph pad software was used for statistical analysis using one-way analysis of variance (ANOVA) and a multiple comparison post-test Tukey for determining statistical significance (set at $P < 0.05$ or $P < 0.0001$) between data sets.

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4.4. Result and Discussion

Efficiency of drug encapsulation and stable drug retention in liposomes are strongly correlated to vesicle bilayer characteristics, such as charge distribution and perviousness and the physico-chemical properties of the drug, such as molecular weight, surface charge, solubility and partition behaviour between the hydrophilic and hydrophobic regions, as well as method of drug loading (Morrow et al., 2007).

Numerous methods are available for LUV preparation, such as ether vaporisation methods (Deamer and Bangham, 1976; Schieren et al., 1978), detergent removal methods (Milsmann et al., 1978; Philippot et al., 1985), reverse phase evaporation method (Szoka and Papahadjopoulos, 1978), calcium induced fusion method (Papahadjopoulos et al., 1975), microfluidization method (Gregoriadis, 1990), and freeze-thaw method (Pick, 1981). However, only few of these methods are efficient for scaling up.

The objective of this study was to incorporate the HMT into the photosensitive LUV and to study its release and stability of HMT. In this investigation, a particular lipid composition (DPPC:Chol:Bis-Azo PC 16:2:1) was selected to obtain lipid bilayers rigidity necessary to avoid drug leakage after encapsulation while conferring photo-release property upon UV irradiation. DPPC has phase transition temperature of 41 °C which is unfavourable for liposomal stability; the inclusion of 2 µmoles of cholesterol should give the stability and eliminate the phase transition of liposomes (New 1990) while the addition of 1 µmoles of Bis-Azo PC should result in photosensitive liposomes (Sandhu et al., 1986).

4.4.1. Effect of large unilamellar vesicles preparation method on vesicle size, zeta potential and drug entrapment efficiency

In order to evaluate the drug entrapment efficiency, LUV with or without HMT were prepared by two methods: interdigitation fusion method (Ahl et al., 1994) and extrusion method (Hope et al., 1985). In comparison to interdigitation fusion method, extrusion method is a straightforward process and forms smaller LUV vesicles within a short period of time. However, it requires polycarbonate filters and LUVET apparatus. It has been noted that SUV increase in size, on the other hand remain unilamellar following a freeze-thaw cycle (Hope et al., 1986; Mayer et al., 1985). Therefore, in this investigation prior to extrusion, freeze-thaw procedure was employed in order to improve the entrapment efficiency of LUV.

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4.4.1.1 Effect of large unilamellar vesicles preparation method on vesicle size, zeta potential

Liposome size affects drug encapsulation and thus impacts drug loading capacity of hydrophilic drug substances into the aqueous region (Franzen and Ostergaard, 2012) Physical instability, such as aggregation of lipid vesicles is due to change in the size and charge distribution. Therefore, liposome size and surface charge are very important factors in drug delivery. The mean diameter, size distribution and zeta potential of LUV formulations prepared through interdigitation fusion method are shown in Table 4.1a for an empty IFV and 4.1b for HMT-entrapped IFV. Table 4.2 a empty LUV and b HMT-entrapped LUV illustrates the characterisation of LUV prepared by extrusion method.

Table 4.1a: Effect of empty photosensitive IFV content on vesicle size, zeta potential

MLV				SUV			IFV		
No. of set	Size	Polydispersity index (PDI)	ZP	Size	PDI	ZP	Size	PDI	ZP
	(μm)		(mV)	(μm)		(mV)	(μm)		(mV)
1	8.70 \pm 0.04	2.11 \pm 0.04	-10.08 \pm 0.41	0.18 \pm 4.3	0.257	-10.49 \pm 1.59	10.38 \pm 0.15	2.29 \pm 0.03	-5.53 \pm 2.65
2	8.93 \pm 0.09	2.15 \pm 0.02	-5.61 \pm 1.14	0.10 \pm 5.7	0.37	-5.53 \pm 1.10	10.26 \pm 0.10	2.08 \pm 0.01	-8.96 \pm 1.87
3	8.55 \pm 0.13	2.12 \pm 0.03	-6.37 \pm 3.91	0.15 \pm 1.7	0.266	-4.66 \pm 1.73	11.74 \pm 0.17	2.16 \pm 0.03	-10.37 \pm 0.56
4	8.77 \pm 0.11	2.09 \pm 0.04	-3.79 \pm 1.00	0.10 \pm 9.6	0.35	-4.24 \pm 2.25	10.43 \pm 0.27	2.10 \pm 0.05	-3.05 \pm 2.86
5	8.62 \pm 0.22	2.11 \pm 0.03	-4.28 \pm 2.09	0.13 \pm 6.1	0.332	-6.68 \pm 2.47	10.24 \pm 0.14	2.24 \pm 0.03	-7.03 \pm 3.24
6	8.51 \pm 0.11	2.14 \pm 0.04	-3.60 \pm 2.40	0.15 \pm 8.5	0.186	-4.30 \pm 2.87	10.11 \pm 1.56	2.26 \pm 0.09	-3.42 \pm 2.79

Empty photosensitive IFV: Liposomes composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC. All formulations were prepared by the interdigitation fusion method entrapping the PBS. Results denote the mean \pm SD from at least 3 independent batches.

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Table 4.1b: Effect of HMT-entrapped photosensitive IFV content on vesicle size, zeta potential

MLV				SUV			IFV		
No. of set	Size	Polydispersity index (PDI)	ZP	Size	PDI	ZP	Size	PDI	ZP
	(μm)		(mV)	(μm)		(mV)	(μm)		(mV)
1	8.50 \pm 0.11	2.10 \pm 0.03	-6.28 \pm 3.91	0.15 \pm 5.3	0.272	-5.05 \pm 2.51	10.87 \pm 0.12	2.29 \pm 0.03	-5.53 \pm 2.65
2	8.34 \pm 0.13	2.14 \pm 0.03	-9.20 \pm 1.50	0.17 \pm 4.5	0.306	-9.11 \pm 1.47	10.64 \pm 0.09	2.08 \pm 0.01	-8.96 \pm 1.87
3	9.35 \pm 0.13	2.08 \pm 0.02	-10.42 \pm 1.32	0.18 \pm 4.0	0.267	-10.29 \pm 1.01	11.04 \pm 0.14	2.16 \pm 0.03	-10.37 \pm 0.56
4	8.08 \pm 0.20	2.04 \pm 0.08	-4.28 \pm 0.83	0.15 \pm 4.8	0.204	-4.44 \pm 3.06	10.89 \pm 0.14	2.10 \pm 0.05	-3.05 \pm 2.86
5	8.43 \pm 0.05	2.0 \pm 0.03	-4.74 \pm 1.30	0.13 \pm 6.1	0.272	-4.22 \pm 0.91	10.64 \pm 0.08	2.20 \pm 0.02	-4.39 \pm 1.54
6	8.85 \pm 0.15	1.95 \pm 0.04	-6.58 \pm 5.10	0.13 \pm 2.4	0.094	-6.37 \pm 3.61	10.61 \pm 0.18	2.18 \pm 0.04	-5.92 \pm 1.59
7	8.62 \pm 0.09	1.93 \pm 0.03	-7.77 \pm 5.41	0.15 \pm 9.5	0.111	-7.69 \pm 4.31	10.89 \pm 0.14	2.20 \pm 0.03	-7.11 \pm 6.96
8	8.84 \pm 0.09	1.97 \pm 0.01	-7.95 \pm 1.01	0.15 \pm 4.6	0.327	-4.47 \pm 0.53	10.70 \pm 0.49	2.14 \pm 0.06	-8.26 \pm 5.36
9	8.77 \pm 0.08	1.94 \pm 0.03	-4.31 \pm 1.27	0.16 \pm 7.3	0.376	-7.29 \pm 0.95	10.58 \pm 0.27	2.10 \pm 0.03	-4.54 \pm 4.82

HMT-entrapped photosensitive IFV: Liposomes composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC. All formulations were prepared by the interdigitation fusion method entrapping the PBS. Results denote the mean \pm SD from at least 3 independent experiments.

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Table 4.2a: Effect of empty LUV (obtained via extrusion method) content on vesicle size, zeta potential

MLV				LUV		
No of set	size (μm)	Polydispersity Index(PDI)	ZP (mV)	Size (μm)	Polydispersity Index(PDI)	ZP (mV)
1	8.38±0.09	2.10±0.06	-3.71 ±1.99	1.59±0.26	1.13±0.05	-3.64 ±3.31

Empty LUV: Liposomes composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC. LUV were prepared by extrusion method entrapping the HMT. Results denote the mean ± SD from at least 3 independent experiments.

Table 4.2b: Effect of HMT-entrapped LUV (obtained via extrusion method) content on vesicle size, zeta potential

MLV				LUV		
No of set	size (μm)	Polydispersity Index(PDI)	ZP (mV)	size (μm)	Polydispersity Index(PDI)	ZP (mV)
1	9.66±0.64	3.79±0.22	-6.21± 1.01	1.54±0.03	1.75±0.31	-3.44 ±1.99

HMT-entrapped LUV: Liposomes composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC. LUV were prepared by extrusion method entrapping the HMT. Results denote the mean ± SD from at least 3 independent experiments.

In this study, DPPC:Chol:Bis-Azo PC based liposomes generated by interdigitation fusion method had diameters of 10.34 nm whereas DPPC:Chol:Bis-Azo PC based liposomes generated by extrusion method were 1.5 nm in diameter. The resultant smaller diameter of LUV formed by extrusion method is due to the use of polycarbonate filters (1μm). Although these preparation methods had major impact on the size of LUV, the differences were not statistically significant ($P > 0.05$).

In addition to size evaluation of the liposomes, uniformity and size distribution were also determined. Polydispersity index (PDI) was used as a measure of liposome uniformity. As shown in Tables 4.1a, b and 4.2a, b, PDI of LUV prepared by extrusion method was lower than that of IFV prepared using interdigitation fusion method. This clearly indicates that the LUVs prepared by extrusion method were homogenously distributed in terms of particle size (Bally, 1988).

Zeta potential is another parameter that affect thermodynamic stability of liposomes (Attama and Muller-Goymann, 2007). From Tables 4.1a, b and 4.2a, b, zeta potential of all vesicle formulations was negative. These results are inconsistent with the results obtained by Pincet et al (Pincet et al.,

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1999) and Wiedmer et al (Wiedmer et al., 2001) who demonstrated that liposomes made from pure zwitterionic phosphatidylcholine (PC) carry a negative charge at neutral pH. Since all liposomes in this study were composed of zwitterionic phospholipids (DPPC), photosensitive lipid (Bis-Azo PC) and neutrally charged cholesterol at pH 7, the final outcome of net negative charge on the liposomal formulations could be due to the conformational effects of lipids in the assembly, that is a buffer dependent orientation of the phosphatidylcholine's dipole head group that creates shielding of the positive charge (Jones, 1995; Makino et al., 1991). In theory, this shielding might be reinforced via the cholesterol, where more of the negative side of the dipole sticks-out of the surface of the vesicle and the positive side pulls more into the inside of the membrane due to hydrogen-bonding with the cholesterol-OH group. The phosphatidylcholine's dipole head orientation that is responsible for imparting a net negative charge to IFV can be represented schematically (Fig 4.5) as suggested by Jones (Jones, 1995).

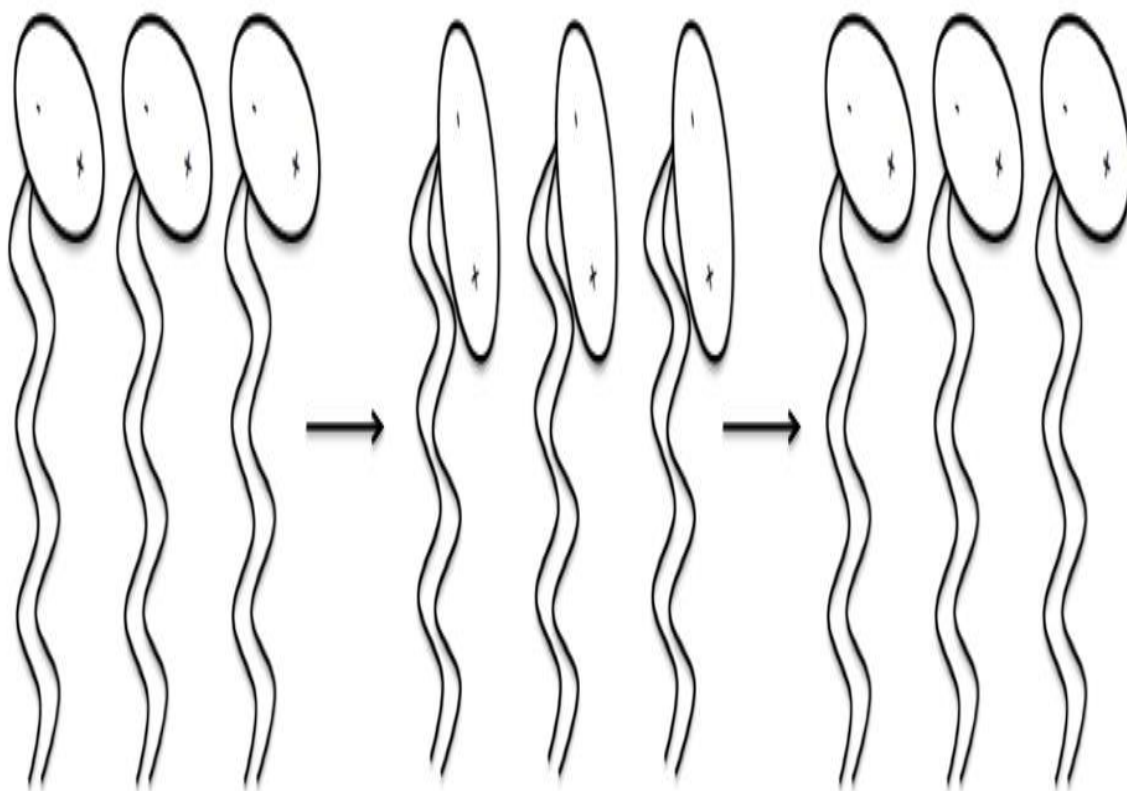


Fig. 4.5: Charge in the orientation of phosphatidylcholine head groups at the surface of IFV.
[adapted from (Jones, 1995; Makino et al., 1991)]

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4.4.1.2. Effect of large unilamellar vesicles preparation method on drug entrapment

Liposomal encapsulation has a considerable effect on the pharmacokinetics and tissue distribution of the administered drugs (Ranade, 1989; Szoka and Papahadjopoulos, 1978; Yavlovich et al., 2011). Therefore, drug entrapment within the system is a key consideration for successful *in vivo* application. Drug loading is entirely based on the chemistry of drug molecules and their partition coefficient (Chimote and Banerjee, 2010). Consequently, water soluble drugs are readily entrapped within the aqueous interior of lipid vesicles. On the other hand, lipid soluble drugs are carried within the lipid bilayer of vesicles. In this study, LUV were used to encapsulate HMT. HMT is a highly water soluble psoralen and hence can get entrapped in the hydrophilic core of liposomes. Following the formulation step, the percent encapsulated HMT in LUV was investigated using UV spectrophotometry. This technique has also been used in the past for quantification of hydrophilic drugs such as capreomycin sulphate and 5-fluorouracil (Giovagnoli et al., 2003; Glavas-Dodov et al., 2003), respectively.

Assessment of encapsulation through UV analysis

In theory, UV analysis is primarily used to quantify the amount of an analyte usually in an aqueous medium. However, in order to perform this, the analyte should absorb in UV/VIS region. Subsequently, Beer-Lambert's Law is used to estimate its concentration (Ingle and Crouch, 1988). HMT is sensitive to UV (Oh et al., 1997). Thus, in this investigation, UV spectrophotometry is used to analyse the HMT content. Previously, Gursoy and co-workers (Gursoy et al., 2004) described UV/visible analysis as a rapid and efficient way of quantifying drug contents.

HMT loading of LUV was determined by measuring the non-entrapped drug presented in the supernatant, following the separation of liposomes via centrifugation. Furthermore, the amount of drug in the supernatant was measured by UV spectrophotometric analysis. The concentration of entrapped drug into liposomes was determined indirectly after centrifugation and separation of the supernatant solution from the resultant pellet. The supernatant was evaluated for the free drug and the amount of encapsulated HMT into LUV was calculated after deducting the HMT presented in the supernatant from the total amount of HMT added initially (this is described in detail in section 4.3.2.5).

As mentioned earlier (in section 4.2), one of the objective of this study is to investigate the encapsulation efficiency of HMT in LUV prepared by two different methods - interdigitation fusion

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method (also referred as Ahl method in this thesis) and extrusion method. The encapsulation efficiency was then determined using the UV/visible spectrophotometry (as shown in Fig 4.6).

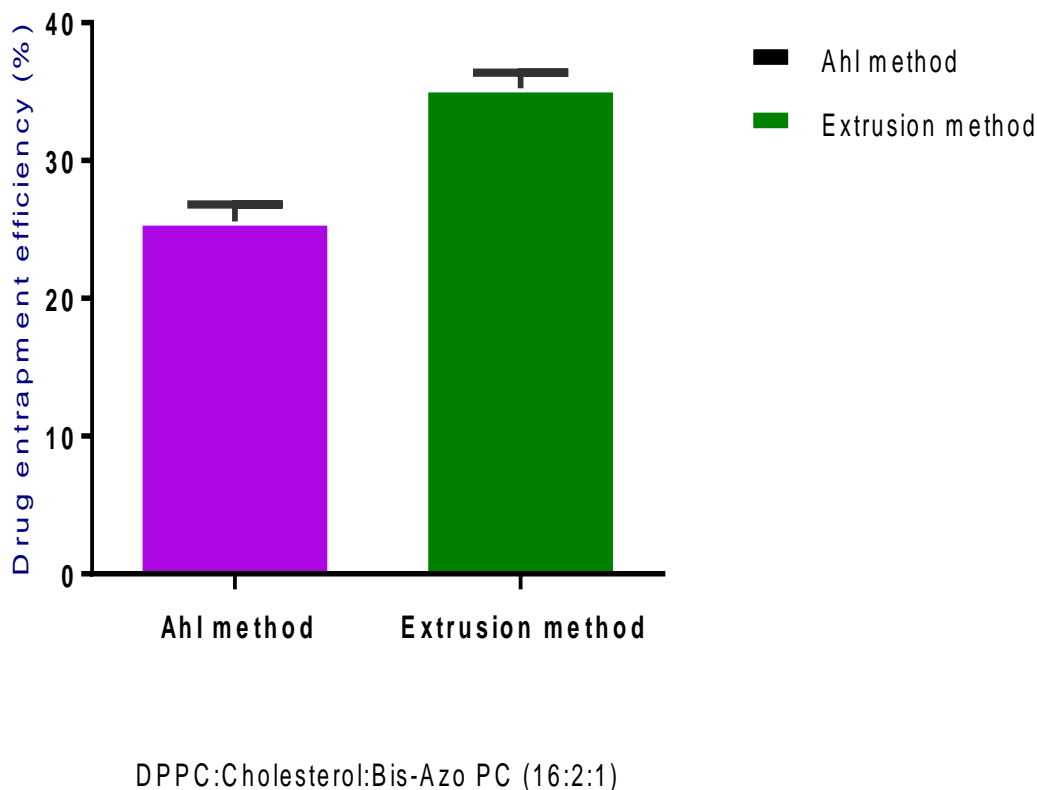


Fig 4.6: Effect of IFV and LUV formulations on drug entrapment.

IFV formulations were prepared by the interdigitation fusion method entrapping the HMT and LUV formulations were prepared by extrusion method entrapping the HMT. All liposomes composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC. The entrapment efficiency of HMT was determined by using UV spectroscopy. The data are expressed as means \pm standard deviation of three independent batches.

It is evident from the Fig 4.6, the entrapment efficiency of IFV prepared by interdigitation fusion method (Ahl et al., 1994) is 25.84 ± 2.02 % while the entrapment efficiency of LUV prepared using extrusion method is 35.1 ± 0.08 %. This means encapsulation efficiency of DPPC:Chol:Bis-Azo PC (16:2:1) liposomes prepared from extrusion method was higher than those obtained by interdigitation fusion method. The results obtained from the interdigitation fusion method is close to the results obtained by Smith and co-workers (Smith et al., 2007). However, Smith and co-workers used calcium chloride as standard drug in their study which is more soluble in water than the HMT. In regard to extrusion method, the results are similar to the LUV prepared by Hope and co-researchers (Hope et al., 1985). However, the trapping efficiencies of LUV could be significantly enhanced by freezing and thawing the multilamellar vesicles prior to extrusion (Mayer

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et al., 1986). In 1998, Messersmith and co-workers (Messersmith et al., 1998) proved that calcium-loaded vesicles prepared using the interdigitation fusion approach were more efficient in encapsulating calcium than those prepared by the extrusion method. Thus, further studies, particularly release and stability studies, were carried out using LUV prepared from interdigitation fusion method.

On the basis of statistical analysis using ANOVA, the results of UV measurements were not significantly different ($P < 0.05$), an indication that similar liposomal formulations prepared through two methods resulted in slightly different entrapment efficiencies.

4.4.2. Morphological analysis of DPPC:Chol:Bis-Azo PC based liposomes

One of the aspects of this study was to use confocal microscopy for morphological analysis of HMT- entrapped LUV. Psoralen is well known as photosensitizer, which makes one's skin and eyes very sensitive to light (Pathak, 1984). There is extensive literature on the determination of two-photon excitation of HMT (Denk et al., 1990; Fisher et al., 1997; Oh et al., 1997). Two-photon excitation has been principally investigated as a method of selectively irradiating a small amount of sample with applications in a three dimensional microscopic imaging and optical information storage (Parthenopoulous and Rentzepis, 1989). Indeed, two-photon excitation of psoralens is beneficial in pharmaceutical applications in which photochemical reactions between two non-covalently complexed molecules that might be initiated in tissues or condensed media ultimately generating the fluorescence and image of the molecule (Oh et al., 1997). In addition, due to the fact that two-photon excitation provides access by visible light to excitation energies corresponding to single UV photon excitation, a whole new class of fluorophores, such as psoralen and several fluorescent indicators, such as Indo-1 for calcium, SBFI for sodium and Mag-Indo-1 for magnesium have become accessible through laser scanning fluorescence microscopy (Haugland, 1989). Based on these principles i.e. two photon excitation of HMT, multiphoton laser system may be used for the determination of HMT-entrapped lipid vesicles. It requires a short duration of pulsed laser system to achieve the high proton flux needed. As a result, fluorescence lifetimes can also be measured in these systems (Gerritsen et al., 1997; Lakowicz et al., 1992). In summary, apart from treating psoriasis, HMT can also be used as fluorescent material in order to visualise the psoriatic skin. To date, little attention has been given on psoralens as fluorescent materials. Therefore, one of the objectives of this study is to detect HMT entrapped LUV through confocal microscopy.

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With the aim of assessing the morphological feature of HMT- entrapped LUV, microscopic analysis was conducted via Leica confocal microscopy in order to scrutinize the membrane structures of the LUV formulations prepared using the two methods i.e. IFV and extrusion methods. As during liposomal characterization, particle sizing via laser diffraction provides the information of liposome size, the application of microscopic imaging analysis can further assure the structural integrity of the liposomes produced (Bibi et al., 2011). In this study, vesicles with or without entrapped fluorescent HMT were prepared using formulations of DPPC:Chol:Bis-Azo PC (16:2:1) and visualized on a Leica multiphoton confocal microscope using an 63 X oil objective as described in section 4.3.2.10. Particle size distribution was not determined by confocal microscopy owing to the difficulty in obtaining a sufficient number of vesicles. On the other hand, the diameters of imaged liposomes may give a sign of vesicle size. Figs 4.7 (a) and (b) illustrate the size and morphology of IFV with or without HMT prepared by interdigitation fusion vesicles method (Ahl et al, 1994) while Fig 4.8 (a) and (b) represent the size and morphology of LUV with or without HMT prepared by extrusion method (Hope et al., 1985).

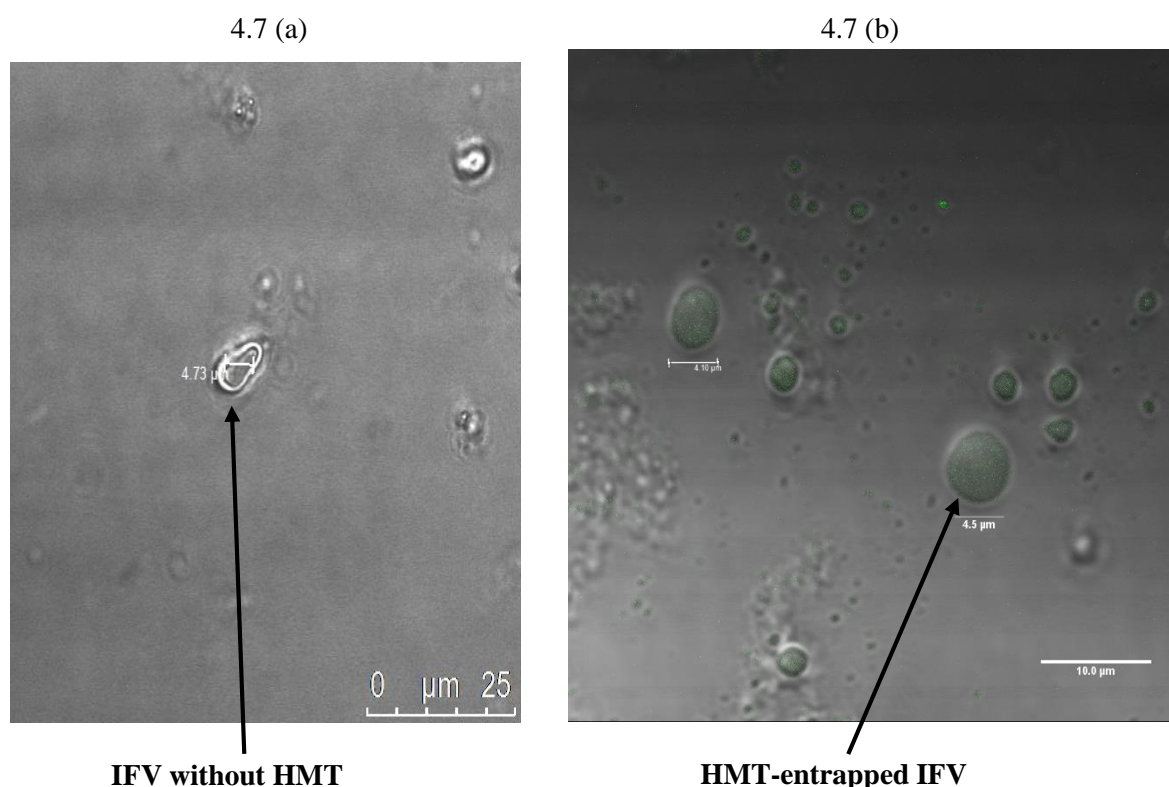


Fig 4.7: Confocal microscopy images of empty IFV (a) and HMT-entrapped IFV (b).

IFV: DPPC:Chol:Bis-Azo PC (16:2:1 μmoles) based IFV with or without HMT taken with a multiphoton Confocal Microscope using a 63 X objective. The green fluorescent marker shown (b) is HMT and is present inside the liposomes i.e. internal aqueous compartment of liposomes.

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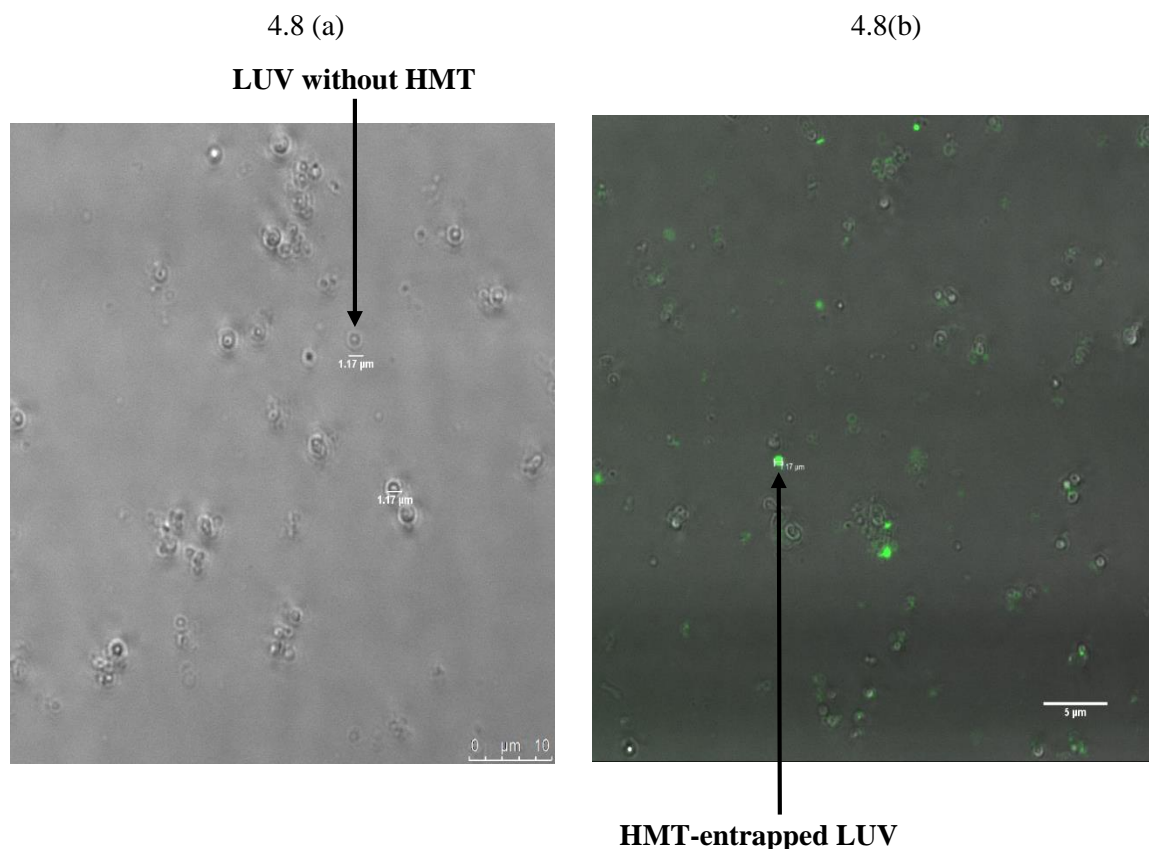


Fig 4.8: Confocal microscopy images of empty LUV (a) and HMT-entrapped LUV (b). DPPC:Chol:Bis-Azo PC (16:2:1 μ moles) based LUV with or without HMT taken with a multiphoton Confocal Microscope using a 63 X objective. The green fluorescent marker shown is HMT and is present inside the liposomes i.e. internal aqueous compartment of liposomes.

It was evident from Figs 4.7 (a) (b) and 4.8 (a) (b) that the particle size of LUV prepared through IFV and extrusion method is ~ 4.6 and 1.17 microns, respectively. LUV prepared by IFV method were substantially larger than LUV prepared by extrusion method. Further, micrographs reveal that all LUV are well-formed vesicles with generally a spherical morphology. Furthermore, HMT can be seen entrapped within the internal aqueous core of the vesicles. Although spherical structures indicative of LUV formation was evident, these vesicles had particle sizes that were greater than a micrometer and thus are in good agreement with corresponding analysis determined via DLS.

Overall, confocal microscopy confirmed the defined morphology and the successful encapsulation of HMT within the LUV prepared via IFV and extrusion methods.

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4.4.3. FTIR analysis

IR spectroscopy was carried out on the obtained products, as this technique can be used to ascertain stability of chemicals in liposomes (Okoro et al., 2014) and the possible interaction between the drug and liposomes (Bensikaddour et al., 2008). The IR spectroscopy generates sample peak that correlate with the frequency of vibration obtained due to the presence of certain function groups and the sample structure can be easily ascertained (Stuart, 1997).

The Infrared absorption spectra of free HMT, empty and HMT-entrapped IFV are presented in Figs 4.9, 4.10 (A) and (B). The spectral analysis of HMT (Fig 4.9) showed characteristic aromatic C-H stretch at 3068.58 cm^{-1} , overtone band at 2919.90 cm^{-1} , C=O stretching of vibration of unsaturated lactones at 1760.79 cm^{-1} , C-C(=O)-C stretching vibration at 1682.56 cm^{-1} , O-C-C band stretching vibration at 1178.20 cm^{-1} , symmetric C-O-C band stretching at 1062.22 cm^{-1} , and plane C-H bending stretching at 758.35 cm^{-1} . The strong band at 3378 cm^{-1} represented O-H stretching in HMT. All these peaks disappeared in the case of HMT- entrapped IFV (Fig 4.10 B), indicating that these groups of the HMT are included in the IFV cavity.

As depicted in Fig 4.10 (A), the spectrum of IFV without HMT showed strong absorbance at 2916.90 and 2849.26 cm^{-1} denoted the C-H stretching, analogous to vibrational stretching in the CH_2 groups of alky chains, with little influence from the stretching vibration in the CH_3 group at 2956.31 cm^{-1} . Even though, the stretching vibration of C-H significantly reduced after HMT entrapment into liposomes, suggesting loading of HMT into the IFV (Fig 4.10 B), the lack of any shift in each peak position after the addition HMT demonstrated that HMT did not chemically change the composition of IFV.

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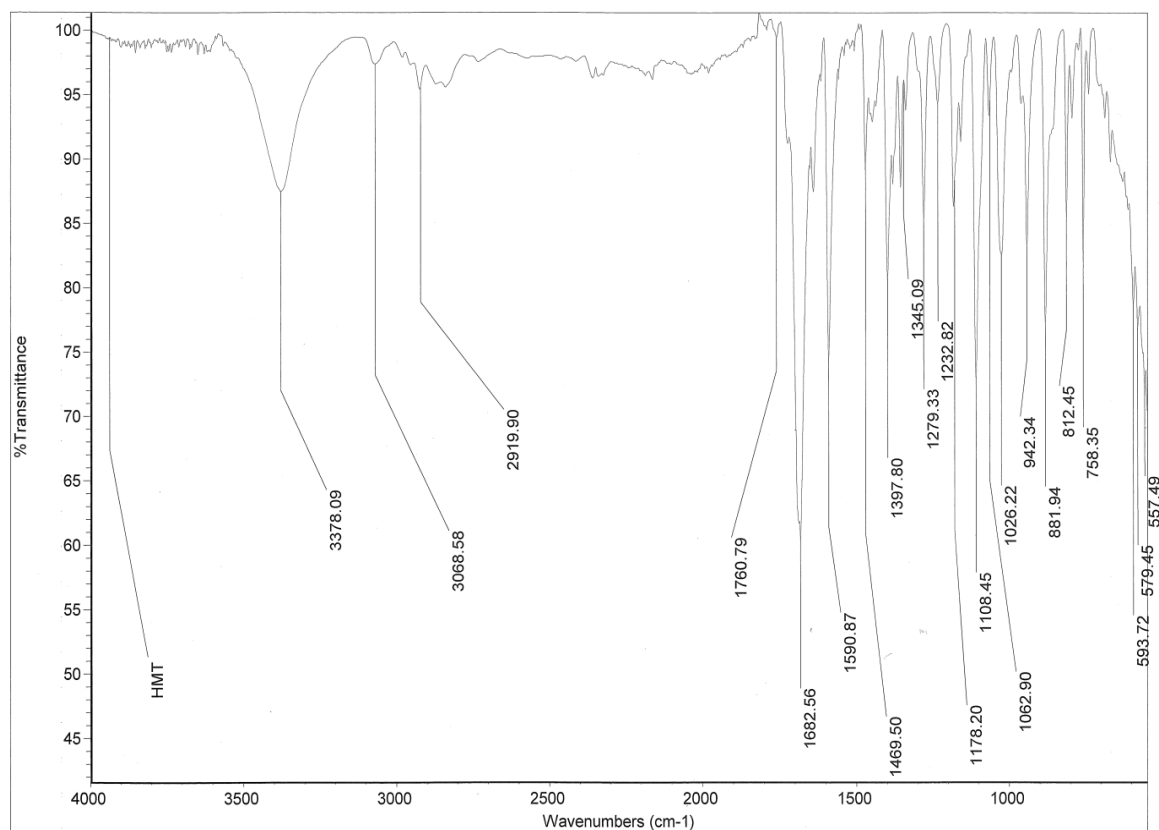
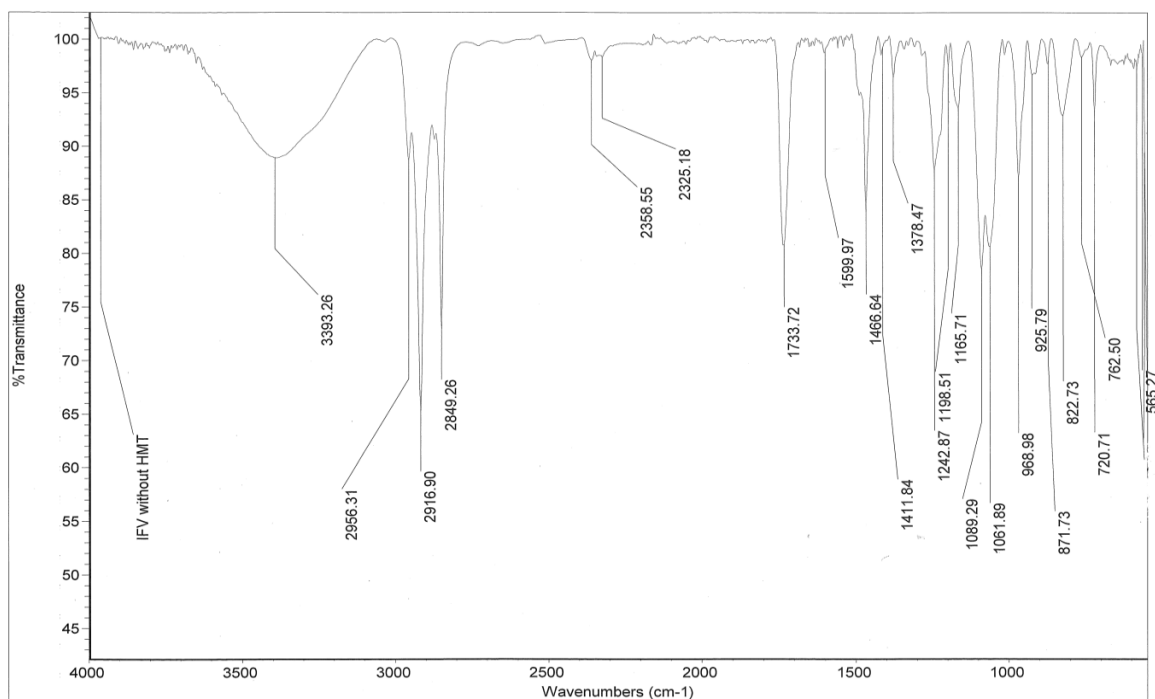


Fig 4.9: IR spectrum of HMT.

Aromatic C-H stretch = 3068.58 cm^{-1} , overtone band = 2919.90 cm^{-1} , C=O stretching of unsaturated lactones = 1760.79 cm^{-1} , C-C(=O)-C stretching = 1682.56 cm^{-1} , O-C-C band stretching = 1178.20 cm^{-1} , C-O-C band stretching = 1062.22 cm^{-1} , C-H bending stretching = 758.35 cm^{-1} , O-H stretching = 3378 cm^{-1} .

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(A)



(B)

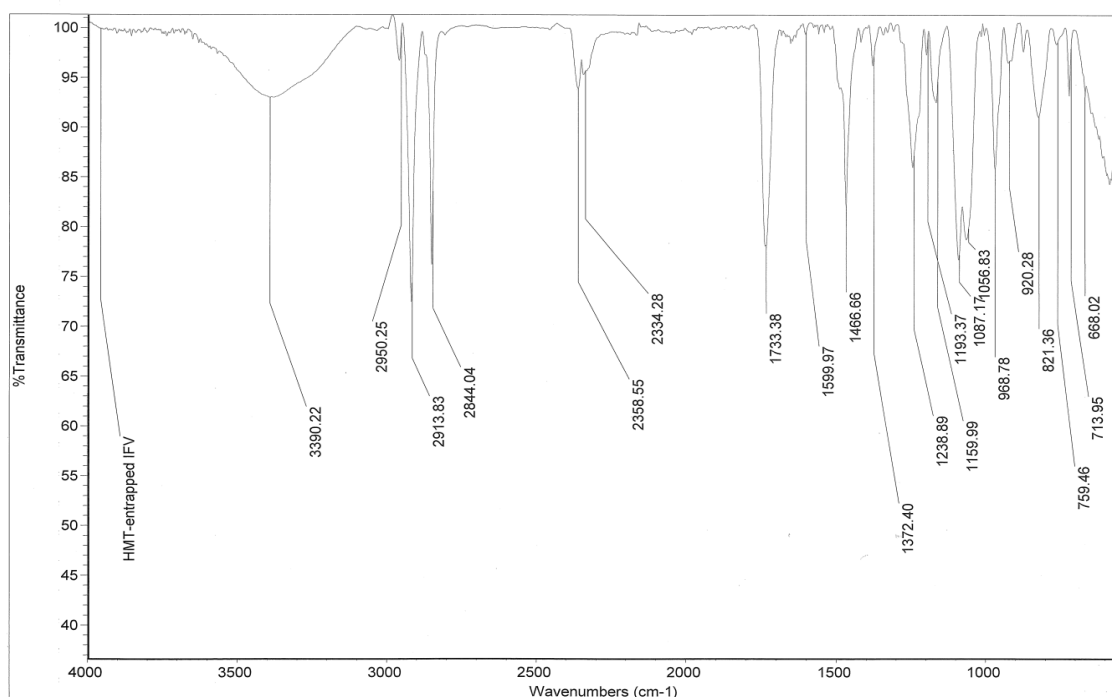


Fig 4.10: Comparison of the FTIR spectrum of A) Empty IFV B) HMT-entrapped IFV.

IFV were generated via interdigitated fusion method. Results denote the mean \pm SD from at least 3 independent batches.

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4.4.4. Photo-induced drug release

A fundamental issue in the assessment of liposomal drug delivery systems is the rate at which the drug is released from liposomes. Drug delivery from photosensitive liposomes is based on disruption of membrane integrity upon exposure to a UV light source (Bisby et al., 2000a). In order to accomplish this, photosensitive molecules (molecules that alter their geometry or conformation upon exposure to UV or Visible light), such as retinoids (Gursel and Hasirci, 1995), azobenzene-based moiety (Hamada et al., 2009) or photochromic lipid Bis-Azo PC (Morgan et al., 1987b) are integrated into the lipid bilayer. Several reports document the mechanisms involved in photoresponsiveness that include photopolymerization of lipid tails (Wang, 2005), azobenzene isomerization (Morgan et al., 1985), cleavage of the lipid tail of plasminogen and diplasminogen through photooxidation (Gerasimov et al., 1999) and a photocleavable NOVC-DOPE (Zhang and Smith, 1999).

Much research in recent years has focused on employing azobenzene derivatives as the photo-control switch. In 1999, Hurst and co-workers incorporated single chain of azobenzene derivatives into liposome which could release drug multi-pulsatilely (Lei and Hurst, 1999). However, it has been documented that single chain amphiphilic azobenzene derivatives encourage the phase separation and fusion of liposomes (Shimomura and Kunitake, 1981; Shimomura and Kunitake, 1987), thus promoting the spontaneous release of liposomes. Earlier Morgan and co-workers (Morgan et al., 1987b) accepted that phospholipid molecules containing the isomerisable group can reduce perturbation to bilayer membranes of single chain amphiphilic azobenzene derivatives. Therefore, they inserted Bis-Azo phosphatidylcholine (Bis-Azo PC) into liposomes as a photo-trigger (Bisby et al., 1999b; Bisby et al., 2000b; Morgan et al., 1995). It should be noted that this photo-triggered system led to a rapid drug release upon UV light irradiation which may not be desirable. However, spontaneous drug release can be controlled by the inclusion of cholesterol. Cholesterol is an essential component in biomembranes and plays major roles in stabilizing the membrane, increasing rigidity of vesicles and reducing the permeability of the biomembranes to water soluble molecules (Ohvo-Rekilä et al., 2002).

In this investigation, light-triggered release was achieved by using photosensitizer i.e. Bis-Azo PC along with appropriate amounts of cholesterol and DPPC. Psoralen, the drug investigated in this study, is very valuable for the treatment of vitiligo and psoriasis (Griffith et al, 2000). It is worth studying whether photosensitive liposomes containing Bis-Azo PC is effective for skin drug delivery as little attention has been paid onto photo-triggered release of psoralen via photosensitive

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liposomes. To demonstrate that these liposomes may serve as promising candidate for light-triggered release of psoralen, optimal liposomal formulation (based on previous protein study in chapter 3) i.e. DPPC:Chol:Bis-Azo PC (16:2:1) was investigated for the release of HMT. The results presented below describe the effect UV light on the kinetics and extent of HMT release from photo-sensitive liposomes.

DPPC (16 μ moles) liposomes containing 1 μ moles of Bis-Azo PC and 2 μ moles of cholesterol were treated with a ROHS 36 w professional UV curing lamp with emission centered at 365 nm for different time intervals i.e. 0, 1, 3, 6, 9, 12 and 15 min at room temp, and HMT release was measured (see section 4.3.2.7) with DPPC:Chol:Bis-Azo PC (16:2:0) liposome as a control. The obtained results are represented in Fig 4.12. Initial drug loading of respective liposomes for various studies are presented in Fig 4.11.

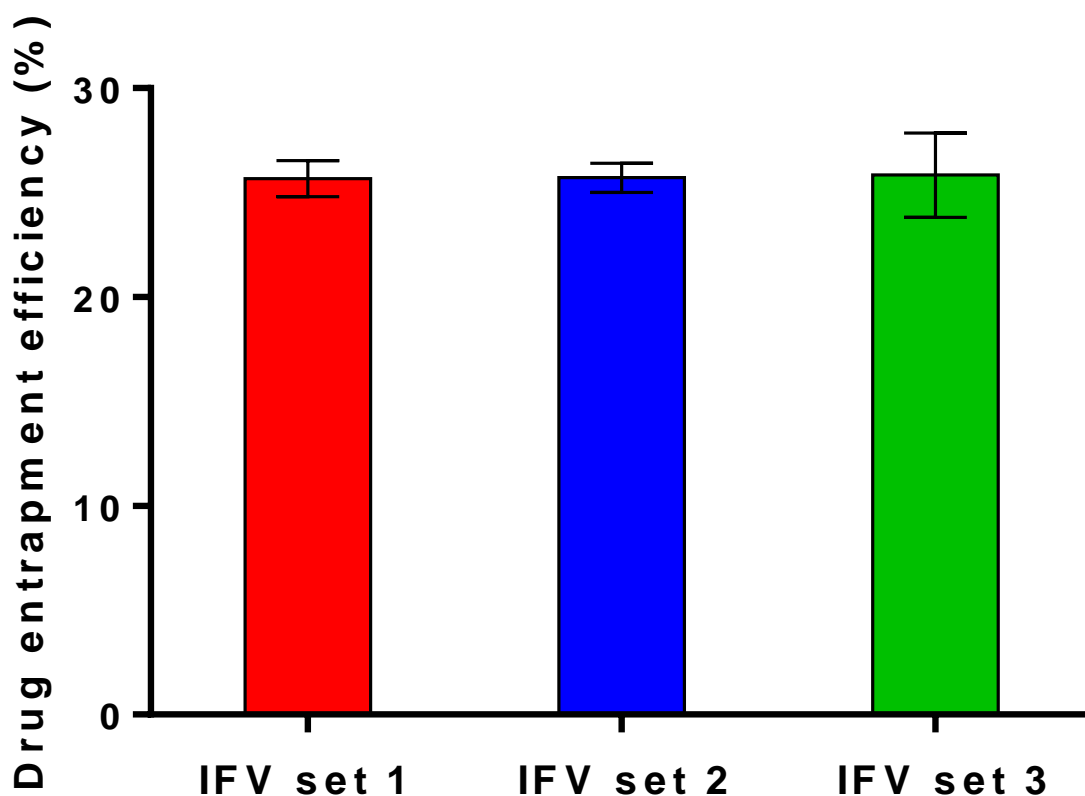


Fig 4.11: Initial HMT loading of liposomes for various studies.

Red column (IFV set 1): liposomes for photo-induced drug study; blue column (IFV set 2): liposomes for short term stability study, and green column (IFV set 3): liposomes for long term stability study. IFV: Liposomes composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC. Results denote the mean \pm SD from at least 3 independent batches.

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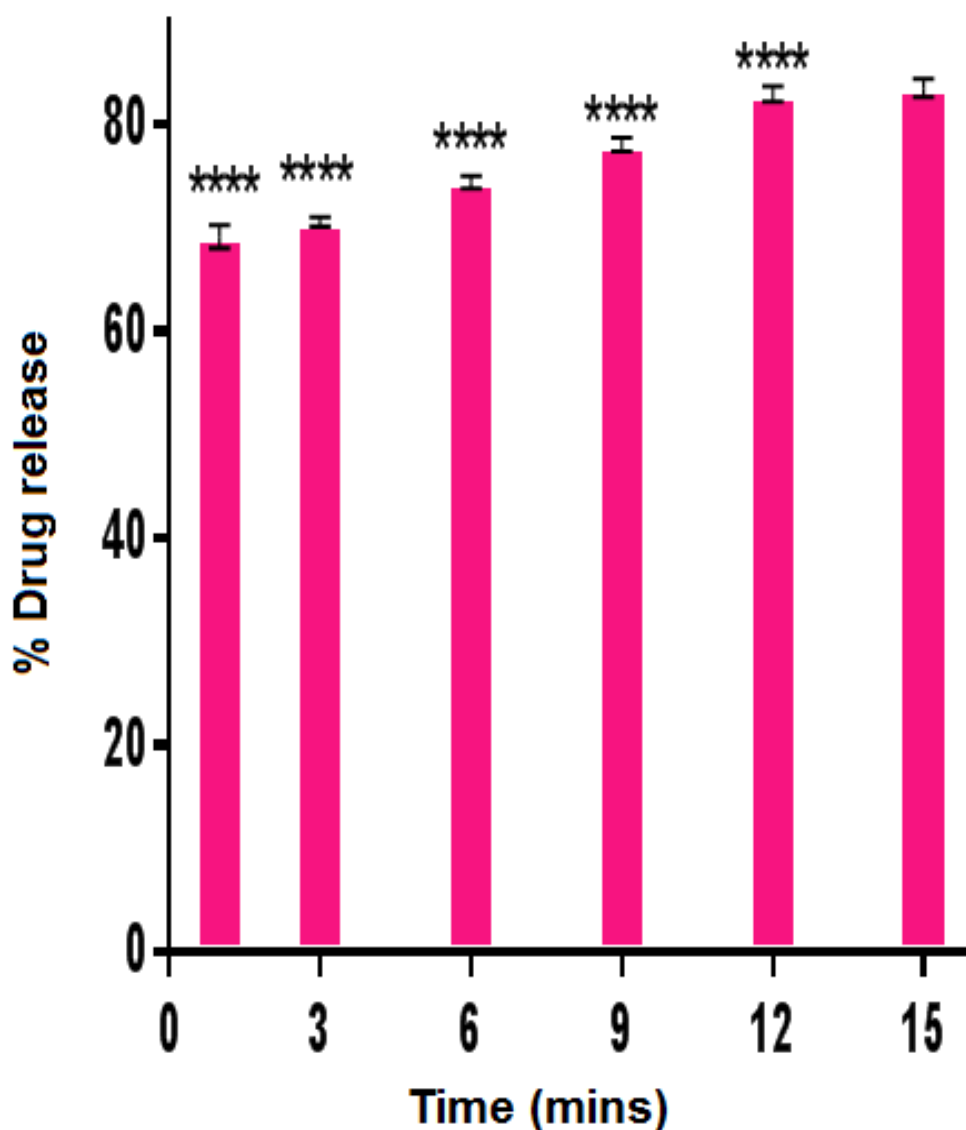


Fig 4.12: Photo-triggered release of psoralen from IFV.

IFV: lipid vesicles were prepared via interdigitated fusion method. IFV composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC. The 100 % releases of HMT were done by ROHS 36w professional UV curing lamp and measured using UV spectroscopy. The data are expressed as means \pm standard deviation of three independent batches. Significance was measured by one-way ANOVA (**** $p < 0.0001$).

The data obtained from this study also shows that no release was observed prior to exposure to UV irradiation (not shown in Fig 4.12), however, after UV exposure, release of HMT from the liposomes occurred (as shown in Fig 4.12). It should be noted that the presence of photosensitive

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lipid Bis-Azo PC in the DPPC liposomes promotes a triggered release of the Drugs (Bisby et al., 1999a). The data presented in Fig 4.12 also show that significant HMT release (up to 70.59 % of total entrapped) occurred from DPPC:Chol:Bis-Azo PC (16:2:1) liposome within 3 min of UV treatment. UV treatment for longer times resulted in 74.46 % and 78.10 % release of HMT after 6 min and 9 min respectively. 83.57 % release of HMT was observed from the liposomes with 12 min UV treatment. However no obvious increase of the drug release occurred after 12 min and 15 min which is considered as maximum leakage of HMT from liposomes. In contrast, control liposomes prepared from 16 μ moles of DPPC along with the 2 μ moles of cholesterol did not promote any substantial HMT release under the identical conditions. This formulation is not sensitive to the UV light at 365 nm and thus no release of HMT was seen after UV irradiation. These results are consistent with results reported by Smith (Smith et al., 2007) who also suggested that threshold concentration of photosensitive lipid Bis-Azo PC was required for the photo-induced release of solutes in response to UV light.

Statistical analyses ($P < 0.05$) shows that the % of HMT release from IFV after 1 min, 3 min and 6 min of UV exposure compared to % of HMT release after 9 min, 12 min and 15 min, as statistically significant ($P < 0.0001$) (Fig 4.12). However, it was also noted from the Fig 4.12 that the % of HMT release from IFV after 12 min UV exposure compared to % release of HMT after 15 min UV exposure was not significant.

Overall, this study supports the notion that a wavelength-specific tunable photosensitizer (Bis-Azo PC) is important for photo-destabilization of DPPC:Chol:Bis-Azo PC (16:2:1) liposomes in the UV wavelength.

4.4.5. Stability assessment

The capability of liposomes to retain their drug load *in vitro* and *in vivo* depends on the liposome construction which can be affected by fatty acid chain length, saturation state of the phospholipids, the existence of cholesterol and temperature (Langner and Kral, 1999). A general sequence of various solutes permeability is: water > > small nonelectrolytes > > anions > cations (Szoka and Papahadjopoulos, 1980). Permeability behaviour of liposomes is highest at the lipid phase transition temperature (T_c), and is generally lower in gel phase compared to fluid phase (Mouritsen and Jørgensen, 1998). As outlined in the introduction section (1.9), the lipid phase transition temperature is influenced by the alkyl hydrocarbon chain length. Therefore, increment in the alkyl hydrocarbon chain leads to stronger van der Waals interaction between the lipids which ultimately

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requires higher energy to disrupt the stable lipid bi-layer, and consequently lipid phase transition temperature increases. Earlier researchers have indicated that the drug retention for liposomes prepared from DPPC lipid ($T_c = 41\text{ }^{\circ}\text{C}$) is higher and more stable than that of liposomes produced from egg PC lipids, $T_c = 0\text{ }^{\circ}\text{C}$ (Chen et al., 2010; Mohammed et al., 2004a). As a result, these stable and strong lipid bi-layers reduce the efflux of drug from lipid vesicles and thus ensuring drug is retained during incubation. Interestingly, numerous experiments have established that cholesterol may be included in various types of liposomes in order to improve bilayer characteristics of liposomes; increasing microviscosity of the bilayers, reducing the permeability of the membrane to water soluble molecules, stabilizing the lipid vesicle and increasing the rigidity of membrane (Demel et al., 1972; Gregoriadis and Davis, 1979b; Semple et al., 1996). According to Papahadjopoulos and co-workers (Papahadjopoulos et al., 1973b), inclusion of cholesterol up to 33 mol % into the liposomes decreases the permeability by eliminating transition temperature of lipid. Another study also reveals that incorporation of cholesterol into liposomes impedes the diffusion of water deep into the lipid membranes and thus enhances the liposomal stability by reducing hydrolytic degradation of liposomes (Simon and McIntosh, 1986). In order to develop successful photosensitive controlled release system, the formulated liposomes must have high retention of the loaded drug in a non-photoreactive environment. Hence, in this study, HMT-entrapped photosensitive IFV was formulated by using the optimal liposomal formulation i.e. DPPC:Chol:Bis-Azo PC (16:2:1) [results obtained from chapter 3]. The stability study of these formulated liposomes was carried out for short- term stability at room temperature and for long-term at $4\text{ }^{\circ}\text{C}$.

4.4.5.1. Short term stability

In this stability study, IFV formulations prepared as described in section 4.3.2.8, were stored in PBS at room temperature for 15 min, 24 h, 48 h and 96 h, respectively. As pointed out by Stark B. et al (Stark et al., 2010), physical and chemical instabilities of carrier systems often limit their application in drug delivery. Therefore, the stored formulations were assessed at the above time courses in terms of their particle size, zeta potential, span and drug retention inside the liposomes. The results are presented in Figs 4.13, and 4.14. No sedimentation was found in any formulations after fresh preparation. Initial drug loading of prepared IFV was approximately 25.84 % (Fig. 4.11).

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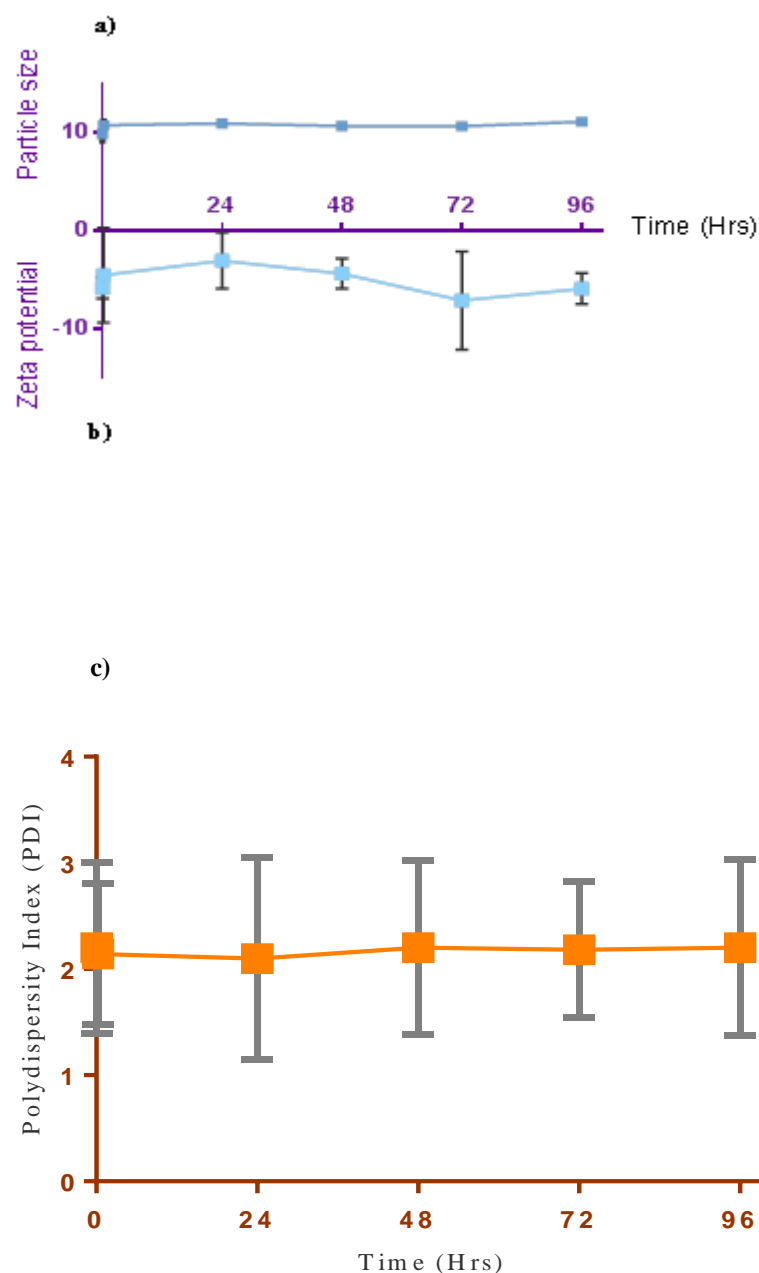


Fig 4.13: Stability assessment of IFV formulation stored at room temperature for 15 min, 24 h, 48 h and 96 h.

IFV: Liposomes composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC. Stability measurements were taken of (a) Size (μ m) and (b) zeta potential (mV) and (c) span measurement after 15 min, 24 h and 48 h at room temperature and analysed via UV spectroscopy. Results represent mean \pm SD of n=3 batches.

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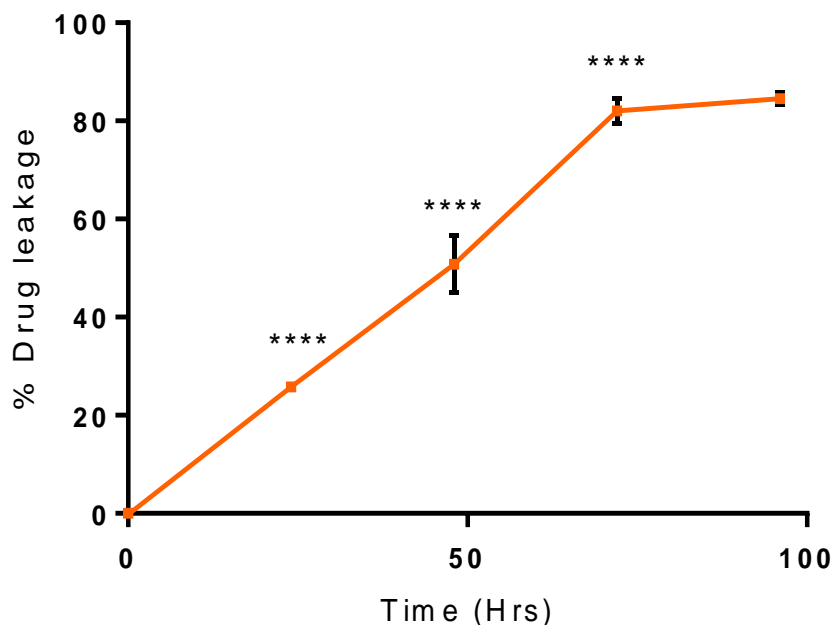


Fig 4.14: Drug leakage (%) from IFV stored at room temp for 15 min, 24 h and 48 h.

IFV: Liposomes composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC. % leakages were taken after 15 min, 24h and 48h at room temperature and measured by UV spectrophotometer. Results represent mean \pm SD of n=3 batches.

According to the results (Fig 4.13 a, b), the formulations remained relatively stable in PBS with regard to size, zeta potential and heterogenicity during the observation period and there was no statistical significance across the assessed parameters over a period of 96 h at room temperature, suggesting that IFV formulations are stable as the storage conditions assessed were not above the phase transition temperature of liposome (Bisby et al., 2000a).

DPPC:Bis-Azo PC IFV containing 2 μ moles of cholesterol stored in the dark at room temperature exhibited no release after 15 min but with leakage of 25.83 % , 50.78 % , 82.08 % and 84.40 % (of total amount entrapped) after 24 h, 48 h, 76 h and 96 h, respectively (Fig 4.14). As can be seen in the figure the percentage release could be a linear manner up to 76 h and the release increased after one day despite the incorporation of cholesterol in the IFV. This is in contrary to the fact that liposomes with neutral charge containing phosphatidylcholines (Yadav AV, 2011) were the most stable and inclusion of cholesterol into neutral liposomes increases the stability of liposomes (Tamba et al., 2004). The inclusion of Bis-Azo PC into the liposome bilayers was shown to remarkably reduce stability thereby limiting the controlled release effect required (Smith et al., 2007).

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Statistical analyses ($P < 0.05$) show that the leakage from the IFV after 24 h is statistically significant ($P < 0.0001$) compared to the protein leakage from the same liposome after 48 h, 76 h and 96 h (Fig 4.14). It can thus be said that statistically the DPPC:Chol:Bis-Azo PC based liposome is not stable after 24 h.

In general, DPPC:Chol:Bis-Azo PC based liposomes size, zeta potential and heterogenicity does not change in PBS stored at 25 °C for a period of 96 h confirming the fact that IFV formulations are stable. However, there is notable drug leakage after 24 h due to the presence of Bis-Azo PC.

4.4.5.2. Long term stability

Long term stability is a factor to be taken into consideration when liposomes are to be applied as a pharmaceutical dosage form. Even though much research in past years has focused on the stability of liposomes, when they were either in solution (Crommelin and van Bommel, 1984; Gregoriadis and Davis, 1979a) or freeze-dried (Fransen et al., 1986; Kirby et al., 1980), little attention has been paid to the leakage of drug entrapped- photosensitive IFV with composition changes of IFV on long term storage. Therefore, in the present study, stabilities of HMT-encapsulated photosensitive IFV (DPPC:Chol:Bis-Azo PC 16:2:1) were assessed by storing it at fridge temperature (4 °C) in the dark for two weeks. The physical (Particle size, size distribution, zeta potential) and chemical (drug retention inside the liposomes) stability of the vesicles are presented in Figs 4.16, 4.17 and 4.18. No sedimentation was found in any IFV formulations after fresh preparation. Initial drug loading was approximately 25 % (Fig. 4.11).

As can be seen from Fig (4.15 a), there was no significant changes in size after IFV formulations was kept at 4 °C for two weeks. Similarly, long term stability study revealed that there was no major changes on the heterogenicity of IFV (Fig 4.15c). In terms of Zeta potential measurement (Fig 4.15b), there was no significant difference in surface charge of IFV comparing one week and two weeks of storage in the dark at 4 °C. These results demonstrate that photosensitive IFV are stable at 4 °C for two weeks. It is worth noting that the enhanced physical stability of HMT-entrapped IFV stored in dark at 4 °C supports the interpretation of the Ghanbarzadeh and Arami results (Ghanbarzadeh and Arami, 2013) who demonstrated that good stability in terms of size, span and zeta potential could be achieved by storing liposomes in fridge at 4 °C.

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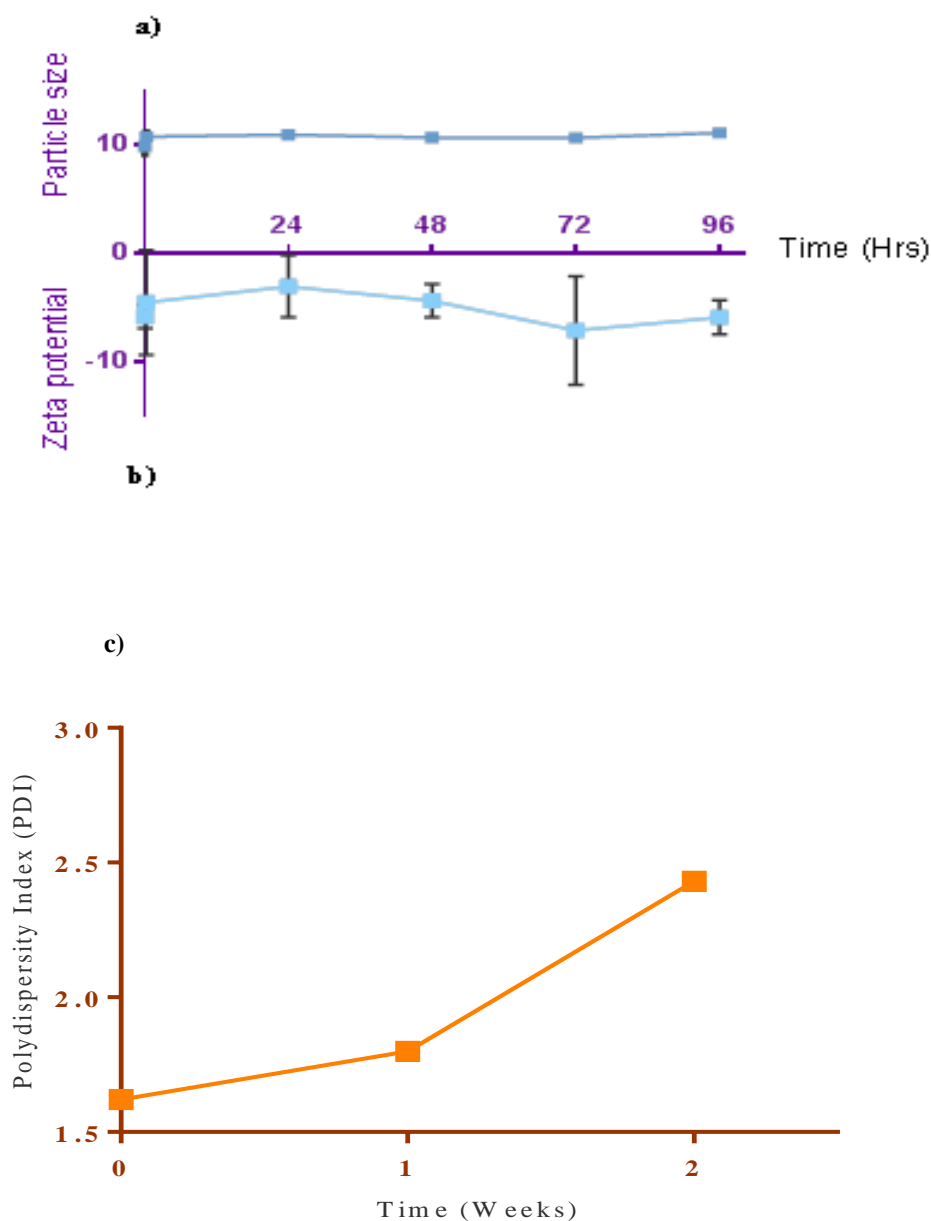


Fig 4.15: Stability assessment of IFV formulation stored at 4 °C for two weeks.

IFV: Liposomes composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC. Stability measurements were taken of (a) Size (μ m) and (b) zeta potential (mV) and (c) span measurement after one week and two weeks at 4 °C and analysed via UV spectroscopy. Results represent mean \pm SD of n=3 batches.

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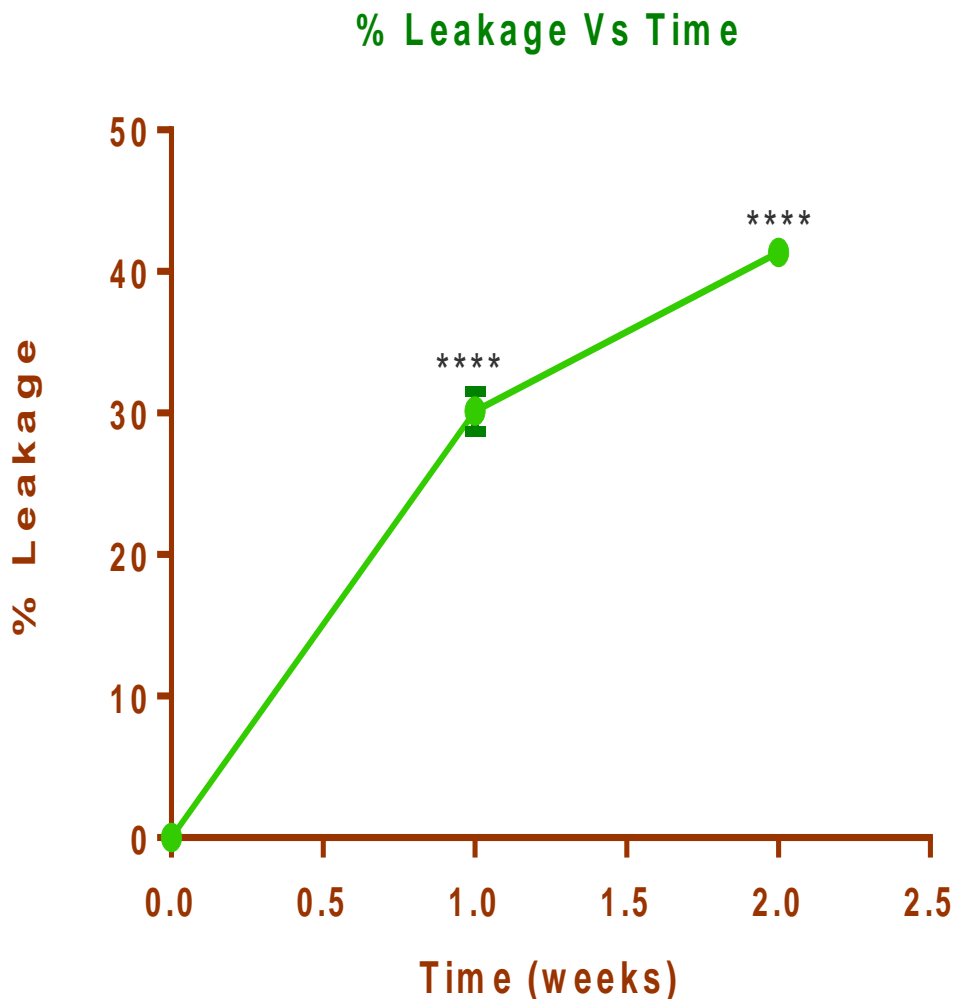


Fig 4.16: Leakage of psoralen from IFV formulation stored at 4 °C.

IFV: Liposomes composed of 16 μ moles of DPPC, 2 μ moles of cholesterol and 1 μ moles of Bis-Azo PC. % leakages were measured after one week and two week at 4 °C and measured by UV spectrophotometer. Results represent mean \pm SD of n=3 batches.

When evaluating the chemical stability of the IFV, it was found out that the percentage of HMT was 33.15 % (of total amount entrapped) after one week and of 41.34 % following two weeks of storage at 4 °C (Fig 4.16). These results are similar to the long term stability studies obtained with BSA-FITC –entrapped IFV (see section 3.4.6.2). These observations are inferred by Freytag (Freytag, 1985) who developed LUV and stored vesicles at 4 °C.

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Statistical analyses ($P < 0.05$) show that the difference in HMT leakage from IFV DPPC:Bis-Azo PC (16:2:1) after one week storage at 4 °C vs protein leakage from IFV DPPC:Bis-Azo PC (16:4:1) after two week storage at 4 °C was statistically significant ($P < 0.0001$). All the HMT release data in Fig 4.16 passed the normality test using one-way analysis of variance (ANOVA) and a multiple comparison post-test Tukey.

On the whole, the short-term and long-term stability studies of the DPPC: Cholesterol: Bis-Azo PC (16:2:1) based IFV showed that higher percentage of HMT retained in formulations stored at 4 °C compared to those stored at room temperature (~25 °C). This data is in consistent with the results obtained by Liu and co-workers (Liu et al., 2002). However, they had developed LUV and indicated that LUV on storage at 4 °C are more stable when compared to the storage at 25 °C. It should be noted that keeping the IFV formulations under refrigerated conditions reduces the liposome instability in terms of psoralen leakage from IFV, even though % leakage of HMT from IFV could be observed. This may be either due to the fact that the presence of Bis-Azo PC in IFV causes higher drug leakage, a phenomenon which was also reported by Smith et al. (Smith et al., 2007) or that the inclusion of 2 μ moles of cholesterol alter the fluidic nature of the membrane and that its behaviour in the bi-layer is dictated by the temperature of the surrounding environment (Chen et al., 2010). However, long-term shelf life of HMT-entrapped photosensitive IFV should be improved by the use of Freeze-drying (lyophilisation) technique that stabilises the lipid vesicles without compromising their physical state or encapsulation capacity (Lasic et al., 1995).

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4.5. Conclusion

The research undertaken was aimed at formulating HMT-entrapped photosensitive liposomes for the triggered release of HMT and further analyse the physicochemical properties and stability profiles of all these liposomes in order to determine HMT retention within liposomal bilayer. This is, to our knowledge, the first time that it has been demonstrated that photosensitive liposomes can be used for the controlled release of HMT, a psoralen derivative that can treat psoriasis condition.

The results from the study of protein-entrapped liposomes (Chapter 3) revealed that IFV prepared from DPPC:Chol:Bis-Azo PC (16:2:1) was optimal for the trigger release of the model protein (BSA). Therefore, similar liposomal composition was used in this part of the project and thus LUV were prepared by two different methods - interdigitation fusion method and extrusion method. Data obtained from the characterisation measurements indicated that no notable difference was observed in physicochemical characterization of DPPC:Chol:Bis-Azo PC (16:2:1) based liposomes, although, the size of LUV was slightly reduced in case of extruded vesicles compared to IFV due to use of polycarbonate filters (1 μ M). Furthermore, detailed examination of DLS demonstrated that PI of all LUV did not show a significant increase. Thus all LUV formulations demonstrated minimum aggregation.

In terms of incorporation studies, it was shown in Fig 4.7 that HMT can be successfully incorporated into LUV (prepared via two methods) containing DPPC:Chol:Bis-Azo PC (16:2:1) effectively. This was further confirmed through confocal microscopy. The microscopic images revealed that all tested LUV were successfully able to entrap the HMT. It should be noted that extruded vesicles encapsulated slightly higher amount of HMT compared to vesicles prepared via interdigitation fusion method. A disadvantage with the extrusion method is that it requires polycarbonate filters and extruder. Therefore, LUV prepared from interdigitation fusion method could be used for further studies.

Photo-triggered release study shows that rapid and controlled release of HMT in a programmed fashion can be achieved by using Bis-Azo PC sensitized LUV. However, the release of entrapped drug is again dependent on the amount of cholesterol and lipid composition. Photo-induced release study also points out that 100 % release is not achievable. Nevertheless, DPPC:Bis-Azo PC IFV (16:2:1) could have 84 % release after 12 min of UV irradiation. These results are similar to protein results (as described in chapter 3).

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A number of LUV formulations prepared through IFV method were subjected to short-term stability studies up to 96 h and long-term stability studies over two weeks period. These studies demonstrated that no significant change in average vesicle (10.89 μm) formed from DPPC:Chol:Bis-azo PC (16:2:1) IFV at 4 $^{\circ}\text{C}$ over two weeks and 25 $^{\circ}\text{C}$ over 96 h despite the slight enhanced leakage of HMT in general at 25 $^{\circ}\text{C}$ when compared to 4 $^{\circ}\text{C}$. These observations are in line with those observed in protein study (as discussed in Chapter 3) and all photo-sensitive IFV were stable at 4 $^{\circ}\text{C}$.

In conclusion, all liposomal formulations tested showed successful release of HMT after UV irradiation and were stable. Thus, photosensitive liposomes DPPC:Chol:Bis-Azo PC (16:2:1) could potentially be employed as carrier systems for the controlled release of HMT offering a potential new strategy for psoriasis and vitiligo treatment.

CHAPTER 5

To Investigate Rat Skin Permeability by Passing Photosensitive IFV through Franz Cell

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5.1. Introduction

Over the last decade, dermal and transdermal drug delivery has been an attractive alternative to oral therapy, because it avoids hepatic first-pass metabolism, potentially reduces the side effects and boosts patient compliance (Barry, 1983). In addition, transdermal delivery systems are inexpensive and can give release for extended periods of time (up to one week) (Prausnitz and Langer, 2008). In order to treat dermatological diseases, a wide array of vehicles ranging from solids to semisolids and liquid preparations are available. Among these, topical delivery of drugs via liposomal formulations has become increasingly popular due to bioavailability and improved therapeutic effects (Cevc, 1996; Schmid and Korting, 1996). Numerous experiments have demonstrated the potential use of lipid vesicles in dermal applications for skin and eyes conditions (Mezei and Singh, 1983; Schaeffer and Krohn, 1982). Even though the principal function of the skin is to offer a protective barrier (Downing et al., 1987; Potts and Guy, 1992), it is nevertheless important to determine how particles as large as liposomes can diffuse pass through the layers of skin. The permeation of transdermal pharmaceuticals via skin can be measured by *in vivo* and *in vitro* techniques (Baert et al., 2010).

In vitro techniques are principally designed to create experimental method that can predict the penetration kinetics of drugs into the human body *in vivo*, and simultaneously measure skin permeability and metabolic activity of drugs (OECD, 2004). It is generally accepted that *in vitro* experimentation offers a number of advantages over *in vivo* studies, including saving time and money, generating better results with reduced degree of parameter variations (van Ravenzwaay and Leibold, 2004). There is extensive research in the literature on the application of the *in vitro* techniques to important problems in dermatopharmacology (Amann et al., 2015; Hermann et al., 1988; Lee and Maibach, 2006; Newbold and Stoughton, 1972; Ostrenga et al., 1971; Scheuplein et al., 1969).

In general, transdermal absorption measurement can be performed by applying test formulations to the surface of a skin sample, which is mounted as a barrier between the donor chamber and the receptor chamber of diffusion cell. Diffusion cells are of two types 1) static type (Franz, 1975) and 2) flow-through type (Bronaugh and Stewart, 1985). Static diffusion cell sample receptor compartment and replace with new perfusate at each time interval whereas flow-through cells utilize a pump to transfer perfusate through the receptor compartment and collect flux by constantly collecting perfusate. Based on the skin membrane orientation, static diffusion cell can be further subdivided into vertical or horizontal diffusion cell.

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Horizontal diffusion cell possess parallel sized receptor and donor chambers situated on either side of the absorption barrier under assessment (Langguth et al., 1986). The absorption barrier is sited in the vertical order (Fig 5.1). The advantage of this diffusion cell is that air bubbles can be easily removed and continuous agitation of the vehicle ensures the homogeneity of formulation (Durrheim et al., 1980; Galey et al., 1976). A disadvantage of this system is the requirement for large volume of donor solution (Flynn and Smith, 1971). Most of the horizontal diffusion cells also require large section skin membrane which is why this type of systems is not useful for human skin (Chien and Valia, 1984). With this type of cells, pre-treatment of skin membrane is impossible. Therefore these cells are beneficial for assessing the molecule diffusivity via membrane or to study the effect of partition coefficient, and pH on diffusion of a molecule (Southwell and Barry, 1981; Southwell and Barry, 1984).



Fig 5.1: The Horizontal diffusion cell [taken from (Smith, 1997)].

The vertical cell assembly, also known as Franz diffusion cell, was initially projected by Coldman (Coldman et al., 1969). Since then this diffusion cell has been modified by many scientists (Chowhan et al., 1978). Thereafter, Franz was the one who proposed vertical diffusion cell based upon a dumbbell-shaped receiver chamber, commonly referred as Franz diffusion cell (FDC) (Franz, 1975). Franz diffusion cell is widely used in drug delivery systems such as sonophoresis,

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iontophoresis or electroporation, and it requires the immersion of both surfaces of skin membrane that may damage skin due to hydration (Pendlington, 2008). The Franz diffusion cell assembly (as shown in Fig 5.2) is composed of two compartments (with a volume of between 0.5 to 10 mL) separated by artificial membrane or skin membrane (Brain et al., 1998). Even though animal or artificial skin can be used as skin membrane, the preferred option is human skin. Apart from the skin, the cell can also be used with artificial membranes mainly because artificial membranes are inert and do not show any biological variation. Synthetic membranes, which are commonly used for *in vitro* study, are of two types. The primary one is either silicon-based membranes polymethylsiloxane or Carbosil that are lipophilic and can be used as barrier similar to SC whereas the second type, such as cellulose esters and polysulphones, can be used to check the release performance of formulations. These artificial membranes also contain pores, therefore they are termed as porous membranes (Ng et al., 2010)

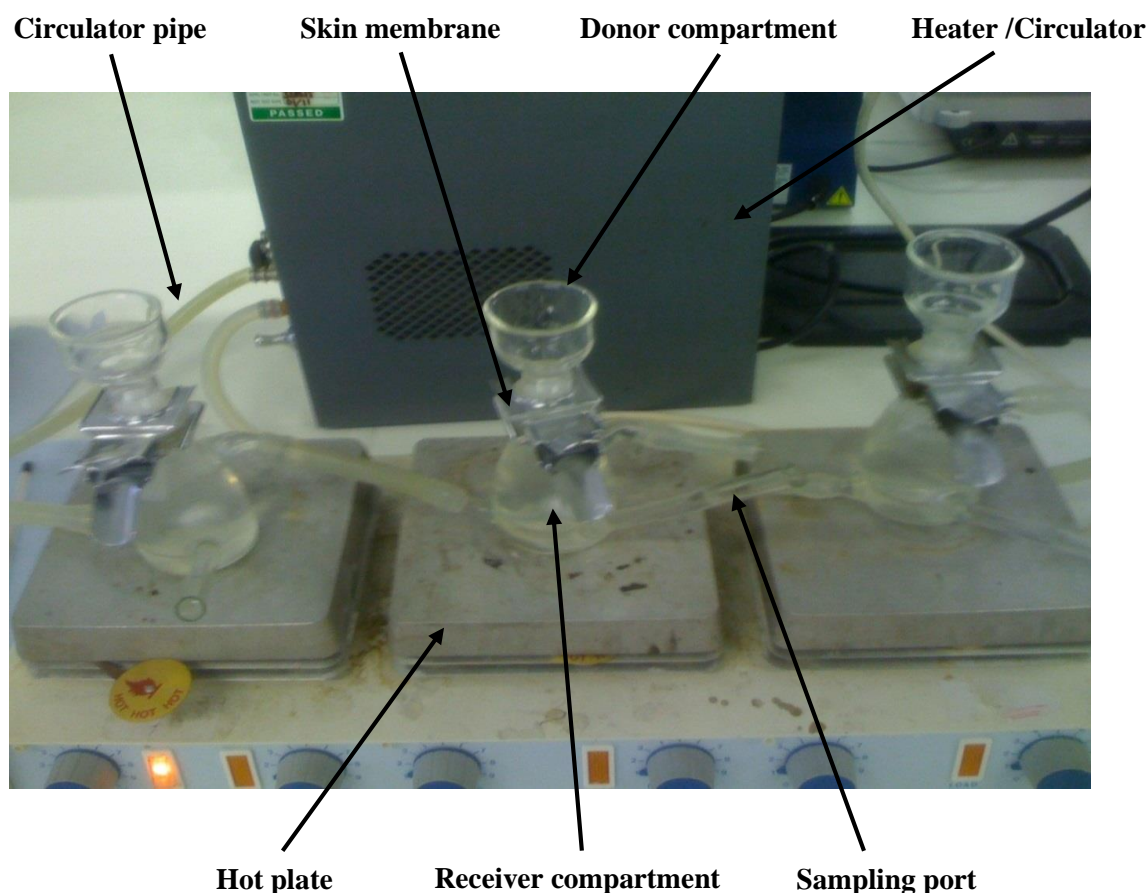


Fig 5.2: Franz cell assembly

In a typical *in vitro* permeation study, both compartments, i.e. donor and receiver compartments are maintained at a constant temperature of 37 °C via a water jacket. A clinically relevant test formulation is applied to the excised skin membranes mounted in FDC through donor compartment. Ideally, the Skin should not be exposed for more than 24 hours as it deteriorates after

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24 hours. The bottom chamber also known as receiver compartment contains diffusion fluid that is continuously stirred, using magnetic stirrer and from which test samples are withdrawn from the sampling port at regular intervals for analysis. These fluid samples are then analysed through the use of various analytical methods such as LC/MS, HPLC, and UV that can determine the amount of active substance which is permeated through the membrane at each time interval. Based on the type of vehicles, the kinetics of drug permeability using Franz cell analysis can vary remarkably (10 to 50 fold). It should be noted that the donor compartment cannot be stirred in this type of diffusion cell; hence active substances must diffuse through the absorption surface.

Both the Franz cell and horizontal diffusion cell possess the advantage that temperature can be readily maintained using water jackets. However, sampling often needs someone to withdraw the aliquots at various time intervals while with traditional flow-through diffusion cell, receptor fluid can be automatically collected and samples can be removed for analysis through scintillation counter, though the traditional flow-through diffusion cells can usually suffer from lack of convenient temperature control. Moreover *in vitro* diffusion cell suffers from evaporation of diffusion fluid from the sampling port and inadequate stirring of the receiver chambers (Chien and Valia, 1984; Gummer et al., 1987). The Keshary-Chien diffusion cell is as an alternative option that gives adequate stirring and the use of stoppers onto the sampling port can reduce the evaporation of diffusion fluid (Keshary and Chien, 1984).

In this investigation, DPPC was used due to the complexity of the skins lipids and it is normal to conduct fundamental experiment on well characterized phospholipid membrane such as DPPC liposomes (El Maghraby et al., 2008). Apart from DPPC lipids, small amount of cholesterol was added for stability and Bis-Azo PC was added in order to make it light sensitive. HMT was selected as potent drug material because it is highly water soluble and few researchers have addressed the evaluation of HMT-entrapped liposomes for topical drug delivery through the use of Franz cell techniques (Fang et al., 2008). For permeation study, rat skin was used. As numerous studies have demonstrated that skin permeability of drugs across the species occurred in the following descending order: rabbit > rat > guinea-pig > mini pig > Rhesus monkey > human (Sato et al., 1991; Wester, 1987). Overall, rat skin is highly permeable (three to five times more) than human skin (Barber et al., 1992). Moreover, Franz cell was also used in order to study the skin permeation of HMT. Moreover, physicochemical characteristics of the obtained DPPC:Chol:Bis-Azo PC based liposomes, such as size, zeta potential, drug loading and viscosity results were investigated. Furthermore, *in vitro* permeation of HMT-entrapped IFVs was performed using Franz cell. Morphological analysis was assessed by using Leica confocal (Confocal laser scanning microscopy).

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5.2. Aim and Objectives

Recently, several attempts have been made to evaluate psoralen-entrapped liposomes for topical drug delivery via using Franz diffusion cell (*in vitro* techniques) (Kumari and Pathak, 2013). However, little attention has been paid to the development of photosensitive liposomes for the delivery of psoralen and subsequently on the evaluation of these liposomes by using *in vitro* techniques. Therefore, the aim of this study was to investigate the permeation behaviour of HMT-entrapped photosensitive IFV through rat skin using Franz diffusion cell. The objectives of this study were as follows:

- To prepare IFV formulations with or without HMT and perform the physicochemical characterization of these liposomes using different parameters such as size, zeta potential, drug loading and viscosity.
- To assess the permeation behaviour of free HMT and HMT-entrapped photosensitive IFV using Franz cell through rat skin and subsequently to calculate skin deposition of free HMT and HMT-entrapped IFV.
- To carry out the morphological analysis of rat skin after Franz cell study via confocal microscopy.

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5.3. Materials and Methods

5.3.1. Materials

5.3.1.1. Chemicals

Chloroform and methanol were purchased from Fisher Scientific, Loughborough, UK. Triton-X; and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich, Poole, Dorset, UK. Distilled water was used throughout. All the chemicals and reagent used were of analytical grade.

4'-(6-Hydroxyhexyloxy) methyl-4, 5', 8-trimethylpsoralen (HMT) (its chemical structure can be shown in section 4.3.1.) was purchased from Berry & Associates, Inc., USA.

5.3.1.2. Lipids

1, 2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol (its chemical structure can be shown in section 3.3.1.2.) were purchased from Avanti Polar lipids, Alabama, USA.

5.3.2. Methods

5.3.2.1. Ethics statement: Rat skin

Female Wistar rats, weighing 500 g, were used and kept in an agreeable environment with free access to rodent diet and water. The rat skin cells were obtained from Female wistar rats killed under PPL 30/3183 (Prof. G L Woodhall). The post mortem rat skin study was approved by the Bioethics Committee at Aston University.

5.3.2.2. Preparation of rat skin (hairless rat skin)

Female wistar rats weighing around 500 g and 3 months old were sacrificed in Aston animal lab. After cervical dislocation, the abdominal and dorsal skin was separated surgically with scissors and scalpel (as shown in Fig 5.3). Hair on the dorsal side of the animal was removed with the help of a shaver, shaving in the direction from tail to head. Dermis part of the skin was wiped 5 to 6 times with a wet cotton swab soaked in ethanol and PBS to remove any underlying fats and muscle tissues. Rat epidermis was prepared by the heat separation method just like human epidermis method (Kligman and Christophers, 1963).

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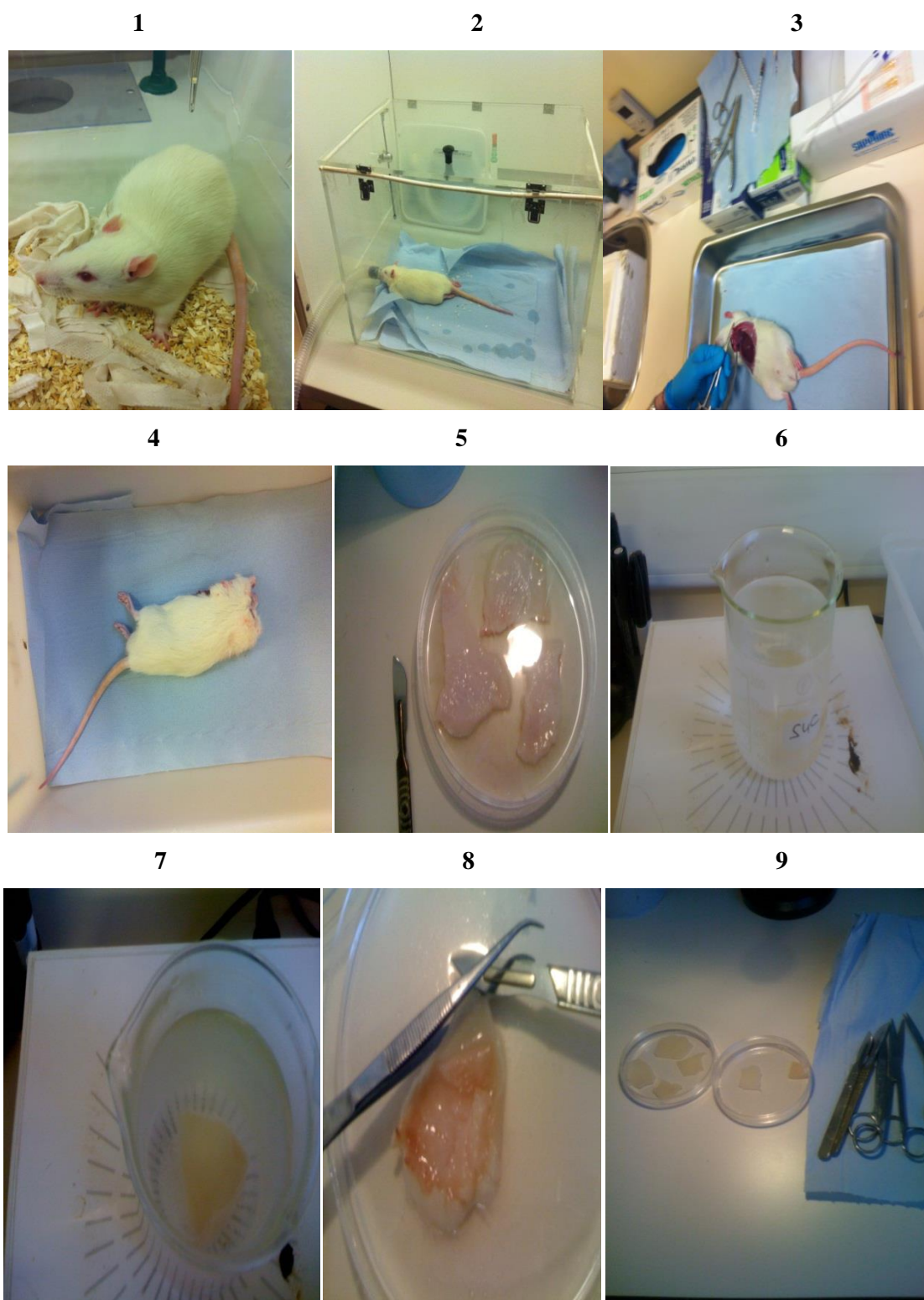


Fig 5.3: Schematic representation of rat skin preparation.

Step1-2: Healthy wistar rat was anaesthetized by using NO and sacrificed; Step 3-4: cervical dislocation, the abdominal and dorsal skin was separated surgically with scissors and scalpel; Step 5-8: rat epidermis was prepared by the heat separation method.

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The full-thickness skin sample was submerged in sterilised water at 60 °C for 3 min. Then the epidermis layer was tactfully separated from the dermis layer with a blunt scalpel. The separated epidermis layer was laid flat and cut roundly into approximately 2 cm in diameter sections before being stored in plastic petri dishes and wrapped in aluminium foils at -22 °C until used.

The thickness of the skin was determined by a micrometre gauge. Aston animal lab uses the same gauge to measure the thickness of tumours.

5.3.2.3. Synthesis of photosensitive lipid Bis- Azo PC

The chemical synthesis of photosensitive lipid 1, 2-bis (4-(n-butyl) phenylazo-4'-phenylbutyroyl) phosphatidylcholine (Bis-Azo PC) was as per the method of Sandhu and co-workers (Sandhu et al., 1986), which is described in Section 2.3.3.4.

5.3.2.4. Preparation of photosensitive IFV by interdigitation fusion method

Photosensitive IFV were generated as described by Ahl and associates (Ahl et al., 1994) which is briefly outlined in section 4.3.2.2.1.

5.3.2.5. Liposome characterisation

Particle size and zeta potential of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT)-entrapped IFV were measured by the same characterization methods as previously used in the HMT study (see sections 4.3.2.3.1 and 4.3.2.3.2).

5.3.2.6. Determination of the initial drug loading

The loading efficiency of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT)-entrapped IFV was determined by removing the un-entrapped 4'-hydroxymethyl-4, 5'-8-trimethylpsoralen with centrifugation at 27,000 rpm until there was no 4'-hydroxymethyl-4, 5'-8-trimethylpsoralen present in the supernatant. Subsequently, the resulting pellet was treated with Triton X-100 to release the entrapped trimethylpsoralen. Briefly, samples were prepared by addition of 500 µL aliquot of liposome suspension to 1 ml solution of phosphate buffer saline followed by addition of 500 µL Triton X-100 (0.1 % solution). Triton X-100 solubilized the liposome membranes and induced release of entrapped contents. All samples were diluted, centrifuged and the supernatant was measured. The drug content of the supernatant was determined by fluorescence spectroscopy (Perkin-Elmer Life Sciences) at excitation and emission wavelengths of 360 nm and 438 nm,

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respectively. A calibration curve was constructed by plotting fluorescence versus concentration of the above standard solutions. Results of regression analyses and the correlation coefficients (R^2) are demonstrated in Appendix-3.

5.3.2.7. Dynamic viscosity measurement

Dynamic viscosity measurements were performed to compare the viscosity of the photosensitive IFV with or without HMT in PBS. Prior to analyses, photosensitive IFV with and without HMT dispersions were generated using the IFV method. Subsequently, the viscosity measurement was carried on Anton Par AMVn automated micro viscometer at room temperature. Approximately 1 mL of liposomal suspension was filled into the glass capillary tube and the measurements were noted at an angle tilt of 70° and -70° . All the viscosity analyses were repeated three times.

5.3.2.8. Skin permeation studies

The Franz diffusion cells (Rasotherm, Germany) were used to measure the percutaneous permeation of free HMT and HMT-entrapped IFV. The Franz diffusion cell is made up of donor and receptor phases, and the skin is attached between them. Prior to the experiments, the receptor and the donor phases were washed with distilled water and acetone. After drying, Franz diffusion cells were filled with 45 mL of phosphate buffer saline (PBS) with a pH of 7.4 as the receptor medium. With the purpose of providing support to the skin fragments, the top cover of receptor phase was covered by silk netting (Silk Fibre, UK) attached to the device with rubber band. Later on, the frozen epidermis skin samples (described in section 5.3.1.3.) were removed and allowed to thaw at room temperature for about 5 min followed by ethanol treatment in order to prevent skin deterioration. Then rounded epithelial skin fragments were gently placed on top of the silk netting attached with previously filled receptor phase of Franz cell so that the epidermis was facing the donor phase. Each cell had diffusion area of 2.49 cm^2 which was measured by Vernier calliper (Chemtool, UK). Then the donor phase was positioned on top, with joint packing in between. A metallic clamp was used to hamper any intrusion of air in the Franz –type vertical diffusion cell. If air bubbles were spotted, the Franz diffusion cell was flicked to manage release of entrapped air bubble. The receptor medium was continuously stirred throughout the experiment by a magnetic stirrer at mode 3. The Franz diffusion cells were connected to a closed circuit circulating water bath (Churchill, England) with a constant temperature of 37°C to keep the skin surface at approximately 33°C . The epithelial skin fragment was equilibrated for 45 min with the receptor cell circulating with water jacket while the donor compartment was dehydrated.

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With the help of plastic syringe, 2 mL of free HMT (10µg/mL) was introduced into the donor phase of each cell. At appropriate time points (30 min, 1 h, 3 h, 6 h, 9 h, 12 h and 24 h) sample aliquots of 1,000 µL were withdrawn from the receptor phase with Hamilton syringe and instantly added an equal volume of fresh receptor medium to the donor compartment in order to ensure sink conditions, and to maintain contact between the skin membrane and the receptor medium. While doing this, bubbles generated below the skin were removed. The sampling ports were enclosed with parafilm and the donor compartments were padlocked with rubber plug with the aim to prevent any cross contamination and vaporization of sample and receptor fluid. Samples taken at various time intervals were analysed by using a Perkin Elmer micro-plate reader. 200 µL of the samples were placed in well of 96 well plate and fluorescence of all these solutions was measured by fluorescence spectroscopy (Perkin Elmer Life Sciences) at excitation and emission wavelengths of 360 and 438 nm, respectively. The whole experiment was carried out at least in triplicate.

Flux of the drug was calculated by using following formulae-

$$Q = (C_n V + \sum_{i=1}^{n-1} C_i S) / A \dots \dots \dots \text{(Equation 5.1)}$$

Q = Cumulative amount of 4'-Hydroxymethyl-4, 5',8-trimethylpsoralen released per surface area of skin (µg/cm²)

C_n = Concentration of 4'-Hydroxymethyl-4, 5',8-trimethylpsoralen (µg/mL) determined at nth sampling interval

V = Volume of individual Franz diffusion cell

$\sum_{i=1}^{n-1} C_i$ = Sum of concentrations of HMT(µg/mL)determined at sampling intervals 1 through n – 1

S = Volume of sampling aliquot, 1 mL

A= Surface area of skin. For this work the surface area was 2.49 cm²

The permeation profile of HMT was obtained by plotting the mean cumulative amount of drug permeated through skin (µg/cm²) versus time. Similarly, *in vitro* permeation of 2 mL of HMT-entrapped IFV (10 µg/mL) was carried out as described above.

5.3.2.9. Determination of skin deposition following permeation studies

Towards the end of 24 hours permeation study, the full skin fragment was taken from the Franz diffusion cell and the remaining formulation which remained on the skin surface was swabbed and

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washed three times with PBS (pH 7.4) and then with ethanol. The same protocol was repeated for three times in order to ensure that no traces of free drug were left onto skin surface. With the intention of extracting drug present in skin, the permeation area of epithelium skin was cut into small pieces using a pair of surgical scissors and the resulting pieces introduced into a vial containing 10 mL of PBS. The resulting suspension was then made uniform by stirring overnight (24 h) and maintained at 37 °C. Subsequently resulting suspension was centrifuged at 27,000rpm for 30 min, and the supernatants were analysed by using a Perkin Elmer micro-plate reader. 200 µL of the supernatants were placed in well of 96 well plate and fluorescence of all these solutions was measured by fluorescence spectroscopy (Perkin Elmer Life Sciences) at excitation and emission wavelengths of 360 and 438 nm, respectively. The whole experiment was carried out at least in triplicate.

Similarly, skin deposition of 2 mL of HMT-entrapped IFV (10µg/mL) was also determined.

5.3.2.10. Confocal microscopy

Following the permeation study, both the photosensitive IFV and HMT treated skin was washed and cross-sectioned by means of scissors. Subsequently, this cross-section skin was mounted onto a microslide. Furthermore, depth permeation of HMT and IFV were examined under Leica confocal microscope at 20× at an optical excitation of 488 nm with argon laser beam as the light source and fluorescence emission was detected above 770 nm. In theory, skin permeation behaviour was examined at multiphoton IR region.

5.3.2.11. Statistical analysis

Graph pad software was used and statistical analysis of the experimental results was performed by ANOVA. Differences were considered statistically significant at $P < 0.05$. All the data values are represented as mean \pm standard deviation of the 3 batches.

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5.4. Results and Discussion

Stability and photo-triggered release data from previous chapter (Chapter 4) showed that HMT-entrapped IFV are stable and give controlled release of HMT after UV irradiation. This is a novel method and can be beneficial in PUVA therapy for psoriasis treatment. However, for successful trans-dermal drug delivery, it is also vital to assess the drug deposition and permeation of HMT-entrapped IFV through skin models. The potential application of liposomes as novel drug carriers for the skin was initially demonstrated by Mezei and Gulasekharam (Mezei and Gulasekharam, 1980) who proved five times greater skin delivery from the liposomes than an ointment containing the same drug concentration. In terms of psoriasis, there is extensive literature on the delivery of antipsoriatic agents to psoriatic skin by employing liposomes (Li et al., 2010; Nagle et al., 2011). As mentioned earlier, little attention has been given on the permeability studies of psoralen-entrapped photosensitive liposomes. Therefore the aim of this chapter was to investigate whether HMT-entrapped photosensitive IFV alter the skin permeability using Franz cell techniques or to synergistically improve the dermal delivery of loaded HMT.

With intention to this, photosensitive IFV were generated by using interdigitation fusion technique (Ahl et al., 1994), as this is a simple and straightforward method. Moreover, it is easy and cheaper than other methods such as extrusion method (Hope et al., 1985), reverse phase evaporation (Szoka and Papahadjopoulos, 1978). It has been shown that the skin drug deposition is dependent on lipid composition (Couarraze, 1995) which can significantly affect the physicochemical properties and drug release profile. Hence, the lipid composition used to prepare photosensitive IFV in this investigation is parallel to those used in the previous chapter (Chapter 4) i.e. DPPC: Chol: Bis-Azo PC (16:2:1).

5.4.1. Characterization of liposomes

DPPC: Chol: Bis-Azo PC (16:2:1) based liposomes with and without HMT were developed using interdigitation fusion method and characterized in terms of size distribution, charge and incorporation efficiency. Particle sizes, polydispersity index and surface charge of the prepared photosensitive IFV with and without HMT are shown in Table 5.1a and Table 5.1b.

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Table 5.1 a) Vesicle size, polydispersity index and zeta potential of various liposomes containing HMT

MLV				SUV			IFV		
No. of set	Size	Polydispersity index(PDI)	ZP	Size	(PDI)	ZP	Size	(PDI)	ZP
	(μm)		(mV)	(μm)		(mV)	(μm)		(mV)
1	6.06 ± 0.01	1.76 ± 0.01	-7.39 ± 1.06	0.10 ± 2.4	0.123	-8.07 ± 3.66	10.44 ± 0.72	2.43 ± 0.23	-9.83 ± 1.89
2	6.77 ± 0.28	2.11 ± 0.04	-10.08 ± 0.41	0.18 ± 4.3	0.257	-10.49 ± 1.59	12.78 ± 0.31	1.95 ± 0.09	-10.57 ± 0.05
3	7.90 ± 0.54	2.34 ± 0.21	-5.61 ± 1.14	0.10 ± 0.5	0.370	-5.53 ± 1.10	10.12 ± 0.48	2.29 ± 0.03	-5.17 ± 4.99

Table 5.1 b) Vesicle size, polydispersity index and zeta potential of various liposomes without HMT

MLV				SUV			IFV		
No. of set	Size	Polydispersity index(PDI)	ZP	Size	(PDI)	ZP	Size	(PDI)	ZP
	(μm)		(mV)	(μm)		(mV)	(μm)		(mV)
1	8.60 ± 0.04	2.11 ± 0.04	-10.08 ± 0.41	0.18 ± 4.3	0.257	-10.49 ± 1.59	10.56 ± 0.10	2.18 ± 0.01	-8.96 ± 1.87
2	8.33 ± 0.05	2.00 ± 0.03	-4.74 ± 1.30	0.13 ± 6.1	0.272	-4.22 ± 0.91	10.79 ± 0.14	2.01 ± 0.05	-3.05 ± 2.86

Photosensitive IFV: Liposomes composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC. All formulations were prepared by the Interdigitation fusion method entrapping the PBS. Results denote the mean \pm SD from at least 3 independent batches.

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It can be seen from Tables 5.1a and b that the vesicle size was found to be around 10 μm . Photosensitive IFV were relatively uniform in size because dynamic light scattering measurements indicated a narrow peak in the particle size distribution. Liposome size has major impact on the transdermal drug delivery, even though no dedicated study was performed until now to shed light on this subject. Several researchers have shown better penetration of hydrophilic drugs from reverse-phase evaporation liposomes than from MLV consisting of egg lecithin vesicles (Gabrijelčič et al., 1994). Apart from this, Esposito and co-workers (Esposito et al., 1998) documented that the permeability coefficient of methyl nicotinate is inversely related to size. Furthermore, Plessis and co-workers (Plessis et al., 1994) studied the influence of liposome size on skin deposition of cyclosporine and they concluded that intermediate sized vesicles rather than small vesicles induced better drug penetration. Based on these findings, it is reasonable to predict the particle size of developed IFV here has a good potential for drug delivery.

As depicted in Tables 5.1a and 5.1 b, the zeta potentials of all photosensitive IFV were negative. As mentioned previously (section 4.4.1.1.) the negative charge is due to conformational effects of lipids in the assembly that is a buffer dependent orientation of the phosphatidylcholine's dipole head group that creates shielding of the positive charges.

5.4.1.1. Drug entrapment efficiency of HMT-entrapped IFV

The amount of HMT entrapped into the photosensitive IFV was also determined. The percentage of entrapment efficiency of all photosensitive IFV was found to be 25 % (Fig 5.4). These results are similar to the results obtained in the previous chapter (Chapter 4).

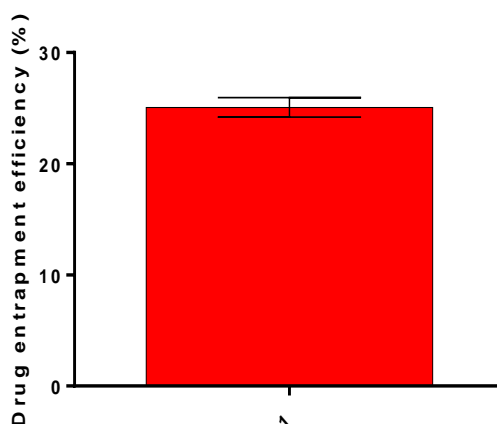


Fig 5.4: Drug entrapment efficiency of HMT-entrapped IFV.

Photosensitive IFV composed of composed of 16 μmoles of DPPC, 2 μmoles of cholesterol and 1 μmoles of Bis-Azo PC. Results denote the mean \pm SD from at least 3 independent batches.

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5.4.2. Viscosity Measurements

To develop photosensitive controlled release formulations, viscosity is a crucial factor to consider. Müller et al documented that the viscous and elastic properties of liposomal dispersions are imperative for their application to skin (Müller et al., 2002).

Therefore, prior to Franz cell study, it is essential to confirm whether there were any difference in viscosity for the IFV with and without HMT (Table 5.2).

Table 5.2: Viscosity of IFV with or without HMT

Formulation	Lipid Composition	Angle [°]	Temp [°C]	Dynamic Viscosity (mPa.s)	Kinetic Viscosity (mm ² /s)
IFV without HMT	DPPC:Chol:Bis-Azo PC (16 µmoles:2 µmoles:1 µmoles)	70.00	25.00	0.76573 ± 0.00335	0.76573 ± 0.00335
HMT-entrapped IFV	DPPC:Chol:Bis-Azo PC (16 µmoles:2 µmoles:1 µmoles)	70.00	25.00	0.75407 ± 0.01895	0.75407 ± 0.01895

IFV: Liposomes composed of 16 µmoles of DPPC, 2 µmoles of cholesterol and 1 µmoles of Bis-Azo PC. All formulations were prepared by the interdigitation fusion method. Each value represents the mean ± SD from at least 3 independent batches.

As depicted in Table 5.2, it was verified that there was no statistical difference in viscosity of photosensitive IFV formulations with and without HMT. This means that addition of HMT into the formulations has minor effect on the viscosity of liposomes. Viscosity may directly impact on the diffusion rate of drug at the miniature level and as per the diffusion coefficient equation, viscosity is inversely proportional to the diffusion rate of the drug (Dash et al., 2013). Therefore, transdermal drug products with moderately high viscosity can exhibit low diffusion rates while compared to transdermal products of comparatively lower viscosity. In this study, addition of HMT into the formulations has null effect on the viscosity of liposomes. As a result photosensitive IFV formulations with or without HMT are ideal for Franz cell work.

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5.4.3. In vitro skin permeation studies

With the purpose of assessing the influence of photosensitive IFV on the diffusion of HMT through skin, *in vitro* permeation studies was carried with stripped Albino Wistar rat skin and Franz diffusion cell device. A previous report indicated that nanostructured lipid carriers (NLC) enhanced the permeation and controlled release of psoralen derivatives such as 8-Methoxypsoralen 8-MOP, 5 Methoxypsoralen (5-MOP) and 4, 5, 8-trimethylpsoralen (TMP) (Fang et al., 2008). The same group also reported that TMP has lower permeation rate as compared to 8-MOP and 5-MOP due to the low water solubility of TMP instigated the difficulty of TMP diffusion from the lipid core. Based on these results, HMT was used in this study because it is highly water soluble due to the presence 4-hydroxymethyl group in the drug TMP.

In the actual investigation, rat skin was mounted in Franz diffusion cell, and a solution of the test formulations such as aqueous solution of HMT (10 µg/mL) and HMT-entrapped IFV (10 µg/mL) was applied to the epicutaneous side of rat skin. Two parameters were obtained: 1) Flux (the rate of permeation) of test formulation via rat skin into the receptor fluid; 2) Deposition or the concentration of formulation in the skin.

5.4.3.1. Determination of flux

The rates of permeation or flux were measured by withdrawing samples from the receptor fluid at various time points over the 24 hours. The concentrations of aqueous solution of HMT (10 µg/mL) and HMT-entrapped IFV (10 µg/mL) in receptor fluid at different times are shown in Table 5.3.

Table 5.3: *In vitro* permeation profiles of HMT through rat kin from dilute HMT solution and HMT-entrapped IFV

Time	Concentration of HMT in receptor cell (µg/ml)	Concentration of HMT- entrapped IFV in receptor cell (µg/ml)
30 min	0.104 ± 0.013	0.090±0.009
1 hr	0.182 ± 0.009	0.176±0.012
3 hr	0.316 ± 0.003	0.307±0.003
6 hr	0.413 ± 0.021	0.402±0.018
9 hr	0.558 ± 0.066	0.510±0.050
12 hr	0.689 ± 0.022	0.642±0.030
24 hr	1.008 ± 0.099	0.990±0.001

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Flux of drug was calculated by using following equation (as noted in section 5.3.2.7)

$$Q = \left(C_n V + \sum_{i=1}^{n-1} C_i S \right) / A$$

The permeation profile of cumulative amount of aqueous solution of HMT (10 µg/ml) and HMT-entrapped IFV (10 µg/ml) in receptor phase at different times over the 24 hours are shown in Fig 5.5.

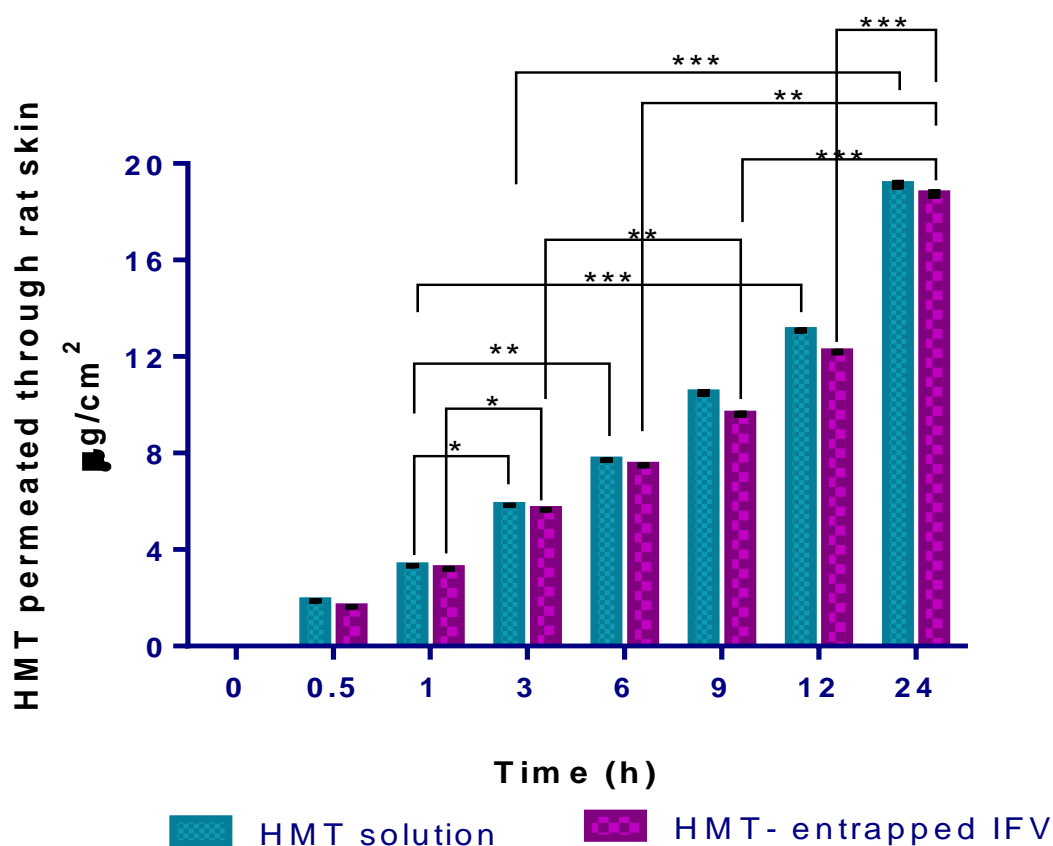


Fig 5.5: *In vitro* cumulative amount – time profiles of HMT through rat skin from dilute HMT solution and photosensitive IFV.

Photosensitive IFV were prepared via interdigitation fusion method. Photosensitive IFV composed of 16 µmoles of DPPC, 2 µmoles of cholesterol and 1 µmoles of Bis-Azo PC. The data are expressed as means ± standard deviation of three independent experiments. Significance was measured by one-way ANOVA (***) p<0.001; ** p<0.01; * p<0.05).

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Table 5.4: Flux analysis

Formulation	Total flux of drug	Skin deposition of drug
Aqueous solution of HMT	19.12 ±0.23 (µg/cm ² /h)	0.881± 0.086 (µg/cm ² /h)
HMT-entrapped IFV	18.74 ±0.11 (µg/cm ² /h)	0.94± 0.01 (µg/cm ² /h)

It is evident from the Fig 5.5 that the skin permeability of HMT from the photosensitive IFV system is nearly the same as that for the aqueous solution of HMT. However, the totals flux of HMT from the aqueous solution of HMT after 24 hours is 19.12 ±0.23 (µg/cm²/h) while the total flux of HMT from the photosensitive HMT after 24 hours is 18.74 ±0.11 (µg/cm²/h) (Table 5.4). It appears that the small variation is due to the rate-determining effect caused by photosensitive IFV. Other studies have demonstrated that the release of hydrophilic solutes (glucose) from the liposomes is the rate–determining step in the overall skin permeation kinetics of hydrophilic solutes (glucose) (Ganesan et al., 1984).

Another research group also validated that hydrophilic materials entrapped principally in aqueous spaces of lipid vesicles have considerably inferior permeation rates to their aqueous solution counterpart (Ho et al., 1985). Therefore, in this study, the skin uptake rates will largely base upon the free HMT concentration that is ruled by the very slow leakage rates from photosensitive IFV. The interfacial transfer of the free drug involves epidermis/water partitioning. In general, all the results are in line with the observations previously reported by Schaeffer and Krohn (Schaeffer and Krohn, 1982) who revealed that the permeation of hydrophilic materials is not enhanced by liposomal delivery.

It is also apparent from the Fig 5.5 that the release of HMT from aqueous solution and photosensitive IFV showed an initial burst that gradually level headed after 6 hours of administration. Surface charge of liposomes also play crucial role in case of permeation. Here, HMT-entrapped IFV showed negative charge (Table 5.1a). It has been reported that formulations with a negative zeta potential are intensely excluded through the skin and thus would result in slower permeation (Fang et al., 2004; Piemi et al., 1999). This could be another reason that the skin

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permeability of HMT from the photosensitive IFV system is lower than skin permeability of free HMT.

Statistical analyses ($P < 0.05$) shows that the percentage release of HMT from free HMT and HMT- entrapped IFV after 1h permeation vs after 3 h permeation was both statistically significant ($P < 0.05$). Moreover, % of free HMT release after 1 h permeation was very significant, ($P < 0.01$) and ($P < 0.001$), to % of free HMT after 6 h permeation and 12 h permeation respectively. Whereas, % release of HMT from IFV, after 3 h permeation vs after 9 h permeation and after 6 h permeation vs after 24 h permeation was very significant ($P < 0.01$). It should be noted that % release of HMT from IFV, after 9 h permeation and after 12 h permeation was very significant ($P < 0.001$) to the % release of HMT from IFV after 24 h permeation.

5.4.3.2. Determination of HMT deposition in the skin

The skin deposition reported in this project refers to 24 hours time point at the end of the experiment and is summarised in Table 5.4. It is conceivable from the results that the HMT deposition provided by photosensitive IFV in epidermis was higher than the free HMT solution which is why total flux of HMT-entrapped IFV is lower than the flux of free HMT.

Taken altogether these results show that HMT-entrapped IFV are better carriers for cutaneous delivery of HMT and this formulation could be useful for treating skin diseases like psoriasis, vitiligo. However, a more extensive study should be undertaken to elucidate the effect of HMT-entrapped IFV topical application and their interaction with the skin. Therefore confocal laser scanning microscopy (CLSM) observations of cross-sections and surface part of epidermis specimens after permeation was carried out by using LEICA confocal.

5.4.4. Confocal microscopy

During the past two decades, Confocal Laser Scanning Microscopy (CLSM) has been widely used as a powerful method to visualize fluorescent materials in the skin. In 1996, Kirjavainen and co-workers documented that the fluorescence from DOPE-(dioleylphosphatidyl ethanolamine) based liposomes was proficient to penetrate deeper into the stratum corneum (SC) compared to that from liposomes devoid of DOPE (Kirjavainen et al., 1996). Later on, in 1998, Van Kuijk-Meuwissen et al proved that the dye applied non-occlusively in stretchy liposomes penetrated deeper into the skin in comparison to occlusive application (van Kuijk-Meuwissen et al., 1998). Furthermore,

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Boderke et al demonstrated in 1997 that CLSM could be a better technique in order to observe the amino peptidase activity in the skin and SC showed considerably less amino peptidase activity than the epidermis (Boderke et al., 1997).

There is extensive literature on the use of CLSM for the purpose of skin studies and skin diseases (Simonetti et al., 1995; Turner and Guy, 1998; Vrhovnik et al., 1998; Zellmer et al., 1998). However, it should be noted that all these researchers used fluorescent dye for labelling of psoralen in order to visualize the psoralen–skin interaction via CLSM. Very few articles are available on the psoralen as fluorescent material. Therefore, in this investigation, psoralen was used as fluorescent marker to examine the epidermis specimens after permeation experiment. The advantage of using IFV containing psoralen as fluorescent marker is as follows –

- It reduces the photosensitive IFV size (10 μm) (as depicted from table 5.1 a) in comparison to the liposome containing fluorescent marker conjugated with the psoralen.
- The use of psoralen as fluorescent label can avoid the problems such as lifetime and quantum yield that arises during the selection criteria of fluorescent marker (Sauer et al., 2011).

Numerous investigations with CLSM on *in vivo* skin cells also stimulate potential applications of two-photon –induced emission and photochemistry in high resolution imaging and labelling of skin cells for basic research as well as clinical research (Wilson, 1990; Rajadhyaksha et al., 1995). Oh et al (Oh et al., 1997) indicated in 1997 that two-photon excitation relative to one-photon excitation of HMT is supposed to boost the depth of penetration and range of cellular targets. Therefore, here Leica confocal microscopy was used to observe the cross-sections and surface part of epidermis specimens after permeation experiment

Fig 5.6 shows representative CLSM photomicrographs of rat skin treated with: dilute HMT solution (a) and HMT- entrapped IFV (b). The images were taken after 24 hours Franz cell permeation experiment. For this study, control sample i.e. IFV without HMT (c) was also employed.

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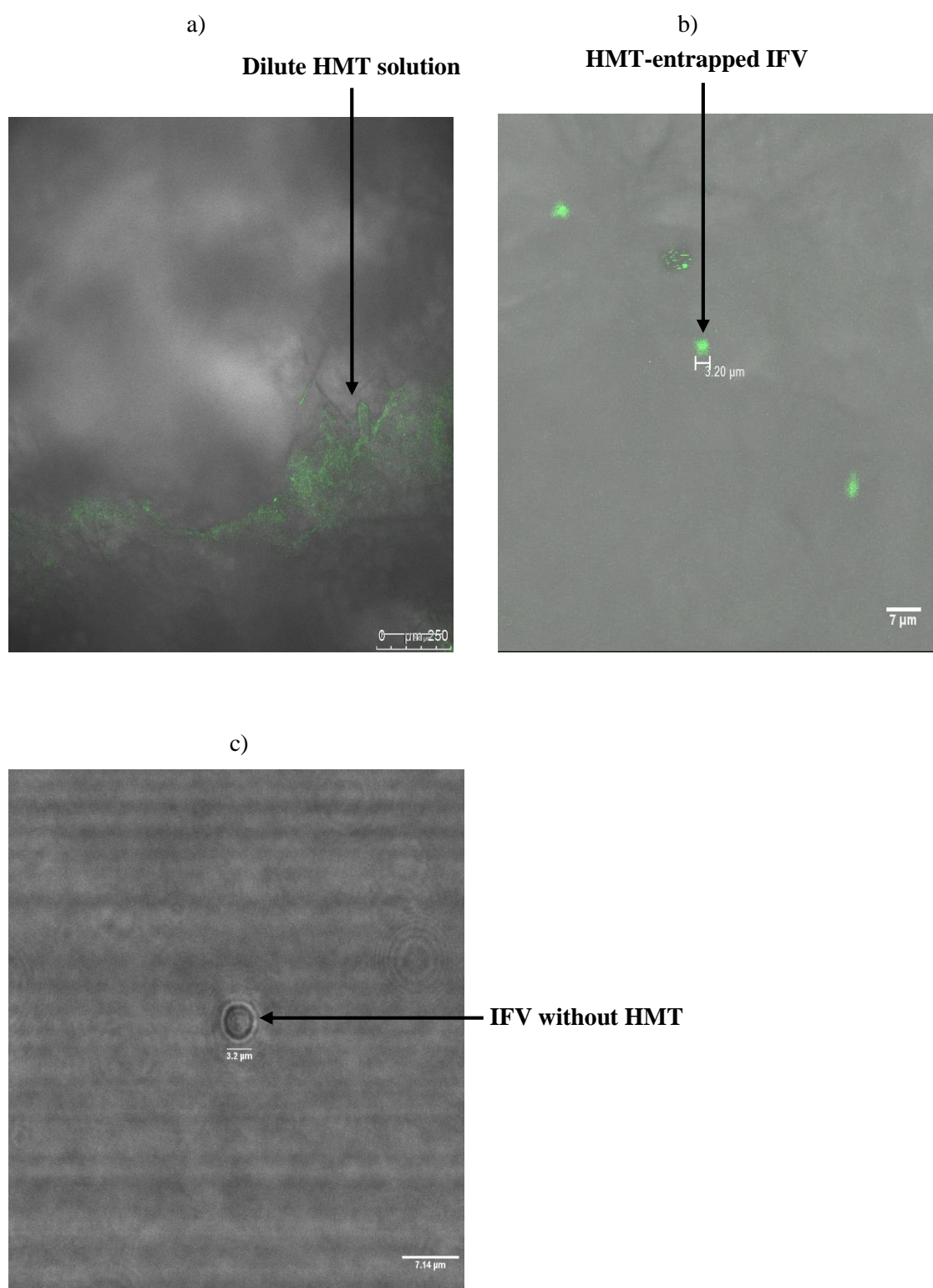


Fig 5.6: The representative CLSM photomicrographs of rat skin treated with various HMT formulations.

a) Dilute HMT solution, b) HMT-entrapped IFV and c) IFV without HMT (control).

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As depicted in Fig 5.6, the average particle size of photosensitive IFV with HMT in the skin is 3.20 μm . Total thickness of epidermis is 7 μm . It can also be seen from the Fig 5.6 that a very high fluorescence was observed in the epidermis with the use of HMT-entrapped IFV as compared to control photosensitive IFV and free HMT. Moreover, in comparison with the skin treated with HMT and empty IFV, cross-sections and surface of the skin treated with HMT-entrapped IFV show increment in hydration.

Overall, these results demonstrated that HMT-entrapped IFV seem to be promising for dermal delivery as they have shown maximum fluorescence in viable epidermis. To date, nobody has used CLSM to visualise photosensitive IFV with HMT. There is extensive research on the use of liposomes containing psoralen conjugated fluorophore in order to visualize the skin permeation (Kalat et al., 2014; Kumari and Pathak, 2013; Nishigori et al., 1998; Zhang et al., 2014). However, few data available on the use of LUV containing psoralen conjugated fluorophore in order to visualize the skin permeation. They have used other psoralen derivatives except HMT. Furthermore no report has used photosensitive IFV. Here, in this study, it was the first time that photosensitive IFV (prepared via interdigitation fusion method) containing HMT was used.

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5.5. Conclusion

Skin conditions such as psoriasis and vitiligo often hark back and are rarely cured through topical drug treatment alone. Hence physicians might suggest PUVA therapy in which a combination of psoralen drugs and long-wavelength UVA light is employed to cure the condition. However, topical application of psoralen itself causes skin irritation. Therefore, it is very important to develop effectual vehicles for delivering psoralen to treat psoriasis condition, which can avoid or minimise the side effects. Photosensitive IFV were developed in this investigation with this aim in mind. Based on the results from chapter 4, it was confirmed that HMT-entrapped photosensitive IFV are stable and readily give controlled release of HMT with UVA irradiation. Apart from this, it is vital to examine the skin permeation of these liposomes. As a result, here, photosensitive IFV were formulated and Franz cell was applied to assess the permeation behaviour of the HMT-entrapped IFV.

To investigate transdermal absorption of drug molecules, the most relevant skin membrane is human skin. Because the availability of human skin is limited, animal skin is regularly employed. That is why in this investigation, rat skin was used and penetration behaviour of photosensitive IFV into rat skin was examined.

From the Franz cell results it could be concluded that inference of photosensitive IFV attributed to smaller HMT release from the HMT-entrapped IFV and produced a slight permeation which was not significant as evidenced by slightly lower amount of HMT into the receiver chamber (18.74 ± 0.11 ($\mu\text{g}/\text{cm}^2/\text{h}$) after 24 h) (Fig.5.5) and slightly higher skin deposition (0.94 ± 0.01 ($\mu\text{g}/\text{cm}^2/\text{h}$) in comparison to dilute solution of HMT (Table 5.4). To minimise these difference, one might consider to use penetration enhancer in this type of systems.

CLSM results clearly indicated the penetration behaviour of HMT-entrapped IFV, and demonstrated the use of HMT as fluorescent marker for the *in vitro* skin study. This imaging behaviour of psoralen could be beneficial to probe answers to questions such as why skin conditions are scaly and non-facile for drug delivery in psoriasis. Hence, psoralen can be used for imaging purpose in addition to psoriasis treatment.

Overall, the work undertaken here has given useful insight into the potential of photosensitive IFV as efficient carrier for superior topical delivery of psoralen along with their potential application to reduce toxicity associated with photosensitive psoralen. However, extensive research work is needed in order to establish *in vivo* efficacy and long-term safety profile of psoralen-entrapped liposomes.

CHAPTER 6

General Discussion

Chapter 6-General Discussion

6.1. Summary and implications of results

The aim of this thesis was to develop Bis-Azo PC based photosensitive liposomes that can be used for the controlled release of active ingredients through long-wavelength UVA irradiation, particularly for therapeutic agents such as psoralen for the treatment of skin disorder such as psoriasis, vitiligo and other skin disorders.

Scientists have already demonstrated that the Bis-Azo PC sensitized liposome system via UV irradiation undergoes cis-trans isomerisation (420/360 nm) in a wavelength specific manner resulting in programmed release of trapped drugs (Bisby et al., 1999b; Bisby et al., 2000b; Morgan et al., 1987b; Sandhu et al., 1986). Moreover, researchers in Aston University have also illustrated that Bis-Azo PC based photosensitive liposomes are beneficial for tissue engineering (Smith et al., 2007). By taking into account the fact that Bis-Azo PC sensitized photosensitive liposomes can be potential carriers for the drug delivery, the first part of this research was focused on the synthesis and characterisation of photosensitive lipid i.e. Bis-Azo PC by using a lipid chemistry method. This synthetic approach was encountered with a few troublesome issues, however, photosensitive lipid i.e. Bis-Azo PC was successfully synthesized. Characterisation studies such as NMR, UV, IR and mass spectrometry confirmed the correct identity of Bis-Azo PC molecule.

The second part of this research concentrated on the incorporation of Bis-Azo PC into neutral liposomes (DPPC:Chol) and these liposomes was applied for the controlled release of model protein (BSA-FITC). The method for the formulation of Bis-Azo PC sensitized liposome was interdigitation fusion method (Ahl et al., 1994). With this method, protein-entrapped IFVs with or without Bis-Azo PC were successfully prepared. These IFV were assessed through confocal microscopy and characterisation techniques such as particle size analyser, zeta potential. Confocal microscopy study revealed that IFV are bigger in size than SUV but are smaller than MLV. The stable entrapment of the model protein was feasible as shown through fluorescence spectroscopy. Within this study one of the aims was to quantitatively and qualitatively assess the impact of cholesterol content on the stability of the prepared IFV to deliver model protein as it is well accepted that the presence of cholesterol improves the bilayer stability (Gallova et al., 2004; Li-Ping Tseng, 2007; Ohvo-Rekilä et al., 2002). The optimum cholesterol amount in terms of protein loading, stability and photo-trigger release of protein was then investigated. These studies showed that the content of cholesterol influences the encapsulation efficiency of protein as well as the release of entrapped protein, and ROHS 36w professional UV curing lamp was more effective for the photo-triggered release of the protein than the other UV lamps (Nichia NSHU590EN UV LED lamp and UV curing flood lamp). Photo-induced study also points out that 100 % release is not achievable and DPPC:Chol:Bis-Azo PC based IFV could give a maximum of 84 % protein release after 12 min of UV irradiation. Results also revealed that the inclusion of increasing amount of

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cholesterol reduces the stability of photosensitive IFV. Most notably, photosensitive IFV formulations stored at 4 °C for two weeks remained stable in comparison to photosensitive IFV formulations stored at 25 °C for 96 h. As a consequence from these observations, DPPC:Chol:Bis-Azo PC (16:2:1) based IFV was chosen as candidate for the controlled release of drugs under UVA irradiation.

Further studies investigated the triggered release of the HMT from DPPC:Chol:Bis-Azo PC (16:2:1) based photosensitive liposomes. Psoralene derivative i.e. HMT was selected as a model compound. Skin conditions such as psoriasis and vitiligo can be treated through PUVA therapy in which combination of psoralen drugs and long-wavelength UVA light is employed to treat these diseases. However, topical application psoralen itself causes side effects such as skin irritation. Therefore, it is mandatory to develop effectual carrier for delivering psoralen in order to treat psoriasis and vitiligo conditions with minima side effects. As a result, HMT-entrapped IFV were developed to release HMT via long-wavelength UVA irradiation. Basically, two different methods, interdigitation fusion method and extrusion method, were utilized to formulate HMT-entrapped liposomes. Results obtained from characterisation studies indicated that no significant difference was noted in physicochemical characterization of DPPC:Chol:Bis-Azo PC (16:2:1) based liposomes prepared by the two methods. Nevertheless, the size of liposomes prepared by extrusion method was slightly smaller than the size of liposomes prepared by interdigitation fusion method. Stable entrapment of HMT was achieved with liposomes prepared via these two different methods which was verified through confocal microscopy. For entrapment studies, indirect UV based method was employed for the quantification of HMT. When compared to interdigitation fusion method, the extrusion method requires polycarbonate filters and extruder, which is why liposomes prepared with interdigitation fusion method could be used for further investigations. Photo-triggered release studies were conducted for HMT-entrapped IFV and illustrated that 100 % release was not achievable, though DPPC:Chol:Bis-Azo PC (16:2:1) based photosensitive IFV could have 84 % release after 12 min of UV irradiation. These results are in line with the protein results (as described in Chapter 3). Furthermore, stability studies revealed that no notable change in average vesicles size (10.89 µm) formed from DPPC:Chol:Bis-Azo PC (16:2:1) based IFV formulations at 4 °C for two weeks and 25 °C over 96 h despite the slight enhanced leakage of HMT in general at 25 °C in comparison with 4 °C. These results are also in good agreement with the protein results (as described in Chapter 3).

The final aim of this project was to assess the permeation behaviour of HMT-entrapped photosensitive IFV through the skin using Franz diffusion cell. The single most important characteristic in the design of transdermal/ topical drug delivery is the rate and extent of drug transport across the skin, in essence the flux of drug molecule. Therefore the permeation behaviour of the HMT-entrapped IFV was investigated. Franz diffusion cell assembly was utilised to assess

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the skin permeation of HMT-entrapped IFV. It is generally accepted that the Franz diffusion cell is a powerful tool to study the permeation behaviour of drug molecules. The skin selected for this study was rat skin as the availability of human skin is limited. The data obtained from Franz cell study showed that HMT-entrapped photosensitive IFV attributed to smaller HMT release and produced slower skin permeation as compared to the dilute solution of HMT but not at a significant level. However, it was also observed that the HMT-entrapped photosensitive IFV contributed to higher skin deposition in comparison to the dilute solution of HMT. In order to avoid these differences, one can consider to use of chemical penetration enhancer, such as oxazolidionones, fatty acids, essential oils, terpenoids into the IFV formulations. Furthermore confocal laser scanning microscopy (CLSM) study was conducted to study the permeation behaviour of dilute HMT solution, HMT-entrapped IFV and IFV without HMT after 24 hour permeation experiment. Results revealed high fluorescence in the epidermis with the use of HMT entrapped-IFV when compared with control photosensitive IFV and free HMT. This is due to the fact that HMT-entrapped IFV showed slower permeation and higher skin deposition after 24 hour permeation experiment. Moreover, in comparison with the skin treated with HMT and empty IFV, cross-sections and surface of the skin treated with HMT- entrapped IFV showed increment in hydration. Results obtained from CLSM also demonstrated that HMT can be used as a fluorescent label for the *in vitro* skin study. This imaging behaviour of psoralen could be beneficial to probe answers to questions such as why skin conditions are scaly and non-facile for drug delivery in psoriasis and vitiligo. Thus, psoralen can be efficient not only for imaging purpose but also for psoriasis treatment.

In summary, the work undertaken in this thesis has given useful insight into the potentials of Bis-Azo PC based IFV as a promising “cage” for the controlled release of active ingredients through long-wavelength UV irradiation and particularly psoralen derivatives that could possibly offer a novel method for the treatment of psoriasis and vitiligo.

6.2. Future Direction

Further work that can be conducted in continuation of this research may include:

- Bis-Azo PC based photosensitive IFV might be toxic and/or carcinogenic in humans and animals. Therefore, toxicity and biocompatibility studies should be needed in order to set the safety profile of these IFV.
- In the immediate future, development of pharmaceutical application of these systems as drug delivery systems could be focused on the design of photocleavable lipids that can be used in *in vivo* experiments. In particular, the photocleavable lipids and the products of their photodecomposition should be nontoxic. Another direction for the future photosensitive liposomes research could be the use of two photon absorption phenomena. Near IR light is capable of penetrating the living tissues without significant absorption and scattering and, therefore, may represent a promising new way for *in vivo* photo-induced release of therapeutic agents.
- Knowledge of physicochemical interaction between vesicle bilayer and HMT/model protein is helpful. This could be investigated using Langmuir trough by recording surface pressure isotherm with respect to time.
- During photo-triggered release study, photosensitive liposomes transform from trans to cis through UV irradiation (Morgan et al., 1987b). This is why the possible role of phase separation in the leakage process needs further evaluation and techniques such as calorimetric measurement, NMR data, hyper DSC and freeze-fracture microscopy would be beneficial in this study.
- In PUVA therapy for psoriasis or Vitiligo treatment, utilisation of long-wavelength UVA light may cause tissue damage (Winterfield et al., 2005; Griffiths et al., 2000). For that reason, extensive research work is needed in order to establish *in vivo* efficiency of HMT-entrapped IFV. Alternatively, since it is well accepted that tissue damage in PUVA therapy can be recovered with the use of Vitamin E (Akyol et al., 2002; Jalel et al., 2009), therefore, utilisation of water soluble D- α -tocopheryl polyethylene glycol succinate (Vitamin E TPGS) can be beneficial in PUVA therapy. Hence, photosensitive IFV can be used as carrier to deliver both HMT and Vit ETPGS for better psoriasis treatment.

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- Franz cell work described in Chapter 5 demonstrated that HMT-entrapped IFV attributed to higher skin deposition and a slightly slower permeation as compared to dilute solution of HMT. Therefore, with intention to increase permeation behaviour of these drugs or to modify the barrier properties of the skin, further studies needs to be carried out by adding penetration enhancers and other excipients into the IFV formulations.
- Photosensitive IFV also can be efficient to deliver Vit D in order to avoid skin cancer. As Vit D protect the human skin exposure to sun rays and play a key role in skin cell development and repair.

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Appendix-1

Aim: To establish a calibration curve for quantification of BSA-FITC (protein conjugated with fluorophore)

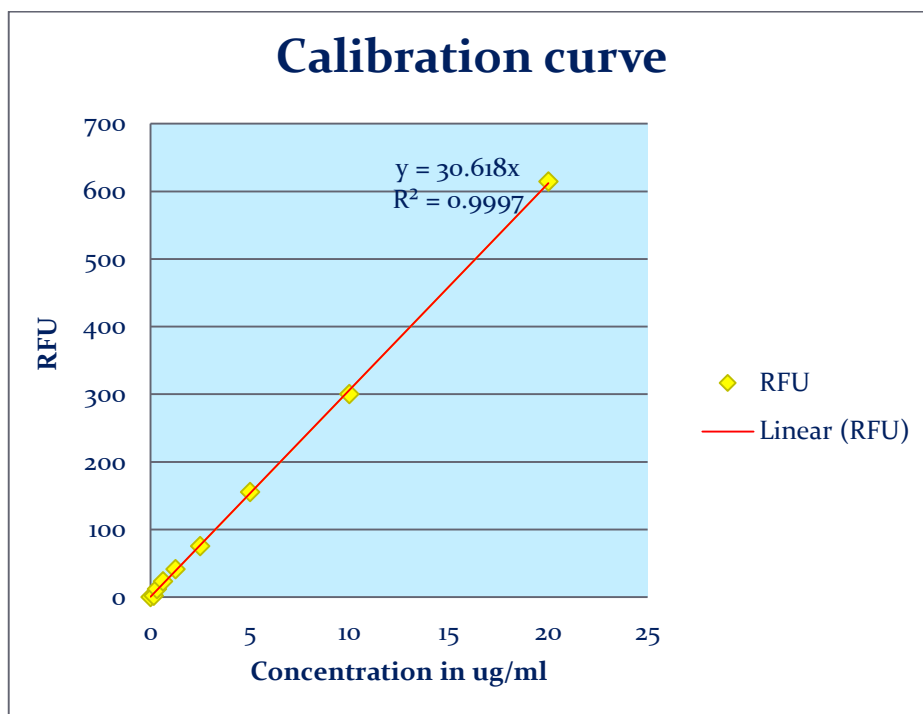
Method:

- A) A standard stock solution was prepared by dissolving 1 mg of BSA-FITC (protein conjugated with fluorophore) in 1 ml of phosphate buffer saline solution at pH 6.8.
- B) A set of standard solutions of BSA-FITC in the range of 0.07 to 20 µg/ml were prepared by the proper dilution of the above stock solution with PBS solution. Further, the fluorescent protein content in the supernatant was determined by fluorescence spectroscopy (Perkin-Elmer Life Sciences) at excitation and emission wavelengths of 490 and 515 nm, respectively.
- C) A calibration curve was constructed by plotting absorbance versus concentration of the above standard solutions (path length to be fixed at 1 cm).

Results:

Conc in ug/ml	RFU	RFU	RFU	Mean	SD
0.07	0.489	0.568	0.693	0.58	0.1
0.1	1.14	1.16	0.9	1.06	0.14
0.125	1.34	1.46	1.73	1.51	0.19
0.15	1.81	1.51	1.96	1.76	0.22
0.31	11.87	12.15	12.49	12.17	0.31
0.625	22.31	22.46	25.07	23.27	1.54
1.25	40.1	42.5	42.1	41.56	1.28
2.5	74.23	76.15	76.5	75.56	1.95
5	154.6	156.4	156.1	155.7	0.96
10	294.5	296.4	296.9	296	1.28
20	600.5	627.1	625.5	615.7	14.9

Absorbance vs Concentration curve



Appendix-2

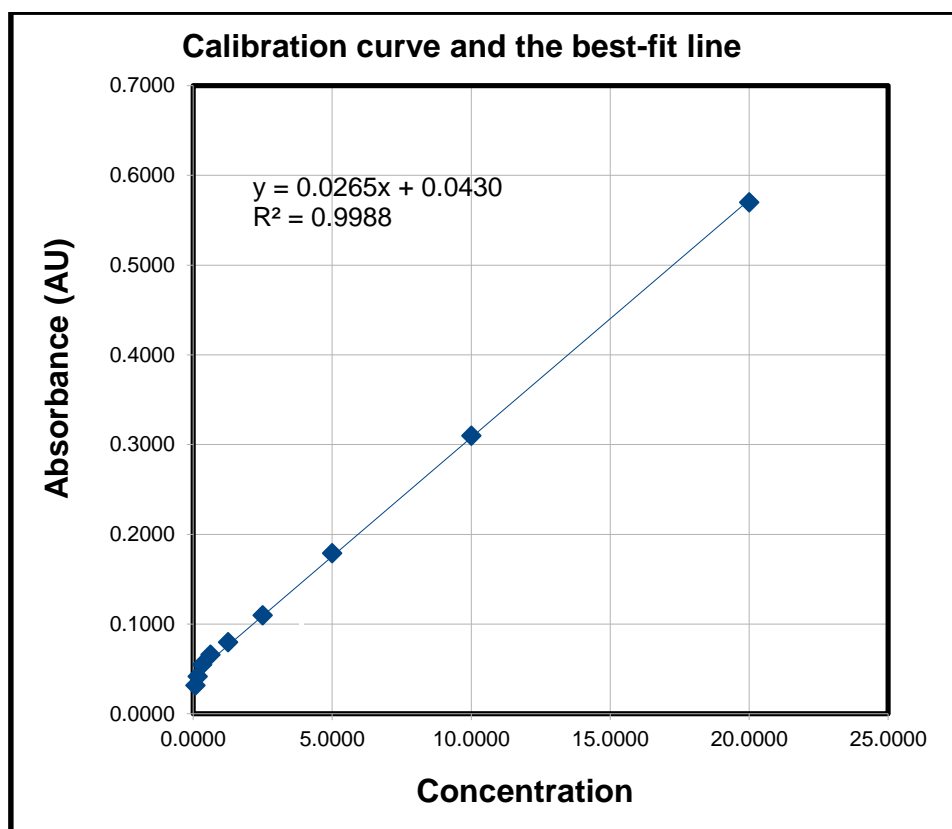
Aim: To establish a calibration curve for quantification of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT).

Method:

- A) A standard stock solution was prepared by dissolving 1 mg of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) in 1 ml of phosphate buffer saline solution at pH 6.8.
- B) A set of standard solutions of trimethylpsoralen in the range of 0.0786 to 20 $\mu\text{g/ml}$ were prepared by the proper dilution of the above stock solution with PBS solution. Further, UV absorbance of all these solutions had been measured by Varian Cary 1 spectrophotometer at $\lambda_{\text{max}} = 249 \text{ nm}$.
- C) A calibration curve was constructed by plotting absorbance versus concentration of the above standard solutions (path length to be fixed at 1 cm).

Results:

Conc in ug/ml	AU	AU	AU	Mean	SD
0.0786	0.0275	0.0321	0.0365	0.032	0.004
0.156	0.0339	0.04193	0.0499	0.041	0.008
0.31	0.0410	0.05517	0.0706	0.055	0.014
0.625	0.0501	0.0601	0.0901	0.066	0.020
1.25	0.0701	0.0800	0.112	0.087	0.021
2.5	0.1000	0.1012	0.1301	0.110	0.017
5	0.1706	0.1626	0.2039	0.179	0.021
10	0.2388	0.3810	0.3129	0.310	0.071
20	0.4998	0.5989	0.5999	0.566	0.057

Absorbance vs Concentration curve

Appendix -3

Aim: To establish a fluorescent based assay for quantification of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) in case of Franz cell study.

Method:

- I. By using Perkin Elmer Instruments, excitation and emission wavelength of 4'-hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) were identified by performing spectral scan (between 250-750nm) in a solution identical to phosphate buffer saline (PBS). 4'-hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) had its excitation λ_{max} at 360 and emission λ_{max} at 438.
- II. A standard stock solution was prepared by dissolving 1 mg of 4'-Hydroxymethyl-4, 5'-8-trimethylpsoralen (HMT) in 1 ml of phosphate buffer saline solution at pH 6.8.
- III. A set of standard solutions of trimethylpsoralen in the range of 0.0786 to 20 $\mu\text{g/ml}$ were prepared by the proper dilution of the above stock solution with PBS solution. Further, fluorescence of all these solutions was measured by fluorescence spectroscopy (Perkin Elmer Life Sciences) at excitation and emission wavelengths of 360 and 438 nm, respectively.
- IV. A calibration curve was constructed by plotting fluorescence (FU) versus concentration of the above standard solutions.

Results:

Conc in $\mu\text{g/ml}$	FU	FU	FU	Mean	SD
0.0708	14.64	14.56	14.49	14.56	0.075
0.156	22.37	22.68	22.97	22.67	0.30
0.31	37.71	39.16	42.10	39.65	2.23
0.625	66.60	67.40	68.80	67.60	1.11
1.25	130.65	115.11	119.87	121.87	7.96
2.5	180.43	195.61	188.51	188.18	7.59
5	264.52	283.38	278.41	275.44	6.77
10	532.15	510.74	521.42	521.43	9.70
20	991.64	995.80	998.20	995.21	3.31

Fluorescence vs Concentration curve

